Investigating the Significance of 11-β Hydroxysteroid Dehydrogenase Type-1 in the Ageing Brain

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Abstract

There has been much research over recent decades into the role of glucocorticoids (GC) in brain function. Much of this has attempted to correlate plasma glucocorticoid with molecular, cellular and behavioural markers. Active GC levels may be modulated locally, in brain cells expressing an enzyme '11β-hydroxysteroid dehydrogenase type 1' (11β-HSD1). This enzyme amplifies active glucocorticoid in intact hippocampal cells *in-vitro* and *in-vivo*. The hippocampus is involved in explicit memory. Its function and an age-related loss of integrity have been shown to be GC related. Absence of 11β-HSD type-1 in transgenic mice, helped to protect aged mice in a hippocampus-dependent task. This thesis was designed to answer questions arising from these findings.

In-situ-hybridisation of the mouse hippocampus and cerebellum, showed no changes in 11β-HSD1 mRNA with ageing. However, decreases in mRNA of corticosteroid receptors indicated a possible, neuro-protective mechanism through changes in GC signalling.

The improved ageing in a hippocampal –dependent behavioural task, was further investigated on a C57BL/6 background. Middle-aged mice showed improved long-term memory in a Y-maze spatial learning test, with no differences in short-term or working memory. In light of the GC role in anxiety and exploration, the elevated-plus-maze and open-field were investigated. There were no definitive differences in visits to arms of the elevated-plus-maze, but an increase in risk assessment suggested increased anxiety in the young Ko (*vs* young control). The young Ko were more active than the young control in the open-field, exploring the outer zone proportionally more than young control, again suggesting increased anxiety, certainly behavioural activation. The significance of enzyme expression in the cerebellum was explored using a motor-learning task. Over 5 days of learning, the young Ko were impaired compared with young controls and there was a negative effect of age.

Although, 11β-HSD1 has been shown as a reductase in hippocampal neurons, there remains some debate over the activity direction in other cells of the brain. To address this, activity was measured in primary, cultured cells from brain regions of interest. No significant activity was found in cells from the frontal cortex, but cerebellar granular

neurons showed reductase activity comparable with hippocampal cultures. There was a positive effect of the presence of glia upon reductase activity.

This study strengthens the hypothesis that 11β-HSD1 contributes to age-related hippocampal deficits, without changes in message. However, there is an increase in anxiety-like behaviour and impaired motor learning. Further research is required, if 11β-HSD1 is to be a pharmacological target in ageing medicine.

Declaration

I declare that this thesis has been written entirely by me and that the work presented here is the result of my own independent investigation, except where otherwise acknowledged in the text. This work has not been and is not currently submitted for any other degree.

Acknowledgements

My first thank you, has to go to my family. Rob and Lykke have sacrificed weekends, holidays and sanity to see this project through. Thank you for all your faith and love. Thank you to my mother for her frequent chats and support, and to my father for his early inspiration. Grandma, you can uncross your fingers now.

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	List of Abbreviations
5-HT	5-hydroxy tryptamine (serotonin)
Ab	antibody
AC	middle-aged C57Bl/6J control
ACTH	adrenocorticotrophic hormone
AEC	3-amino-9-ethyl carbazole
AK	aged 11β-HSD1 129/Ola knock-out or middle-aged 11β-HSD1 C57Bl/6J knock-out
ANOVA	analysis of variance
Arac	Arabinoside C
AVP	arginine vasopressin
AW	aged 129/Ola wild-type
CA	cornu ammonis
CBG	corticosterone binding globulin
CBX	carbenoxelone (3 β-hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate)
DAB	diaminobenzidine tetrahydrochloride
DEPC	diethyl pyrocarbonate
DG	dentate gyrus
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
FDU	fluorodeoxyuridine
g	gram
GABA	γ-aminobutyric acid
GFAP	glial fibrillary acid protein
GMol	granular molecular layer
GR	glucocorticoid receptor
HPA	hypothalamus-pituitary-adrenal
HSD	hydroxysteroid dehydrogenase
ISH	in-situ-hybridisation
kb	kilobase

kg	kilogram
Ko	knock-out
l	litre
LTD	long-term-depression
LTP	long-term-potentiation
M	molar
MAP-2	microtubule-associated proteins
mg	milligram
µg	microgram
µl	micrlitre
MR	mineralocorticoid receptor
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NMDA	N-methyl-D-aspartate
PBS	Phosphate buffered saline
PVN	paraventricular nucleus
SSC	saline sodium citrate
TRIS	Tris (hydroxymethyl) methyl amine
VMN	ventromedial nucleus of the hypothalamus
Wt	wild-type
YC	young C57Bl/6J control
YK	young 11β-HSD1 knock-out
YW	young 129/Ola wild-type

Preface

It would be easy for a researcher in this field to feel that glucocorticoids are omnipotent. It can be a task for the researcher to find a process which is not affected by them. They are described as 'stress hormones', but they have varied and subtle effects throughout our bodies and development. Glucocorticoids have an established role to play in attention (Wolkowitz and Reus 1999; Ellenbogen et al. 2002), mood (reviewed in Holsboer 2000) and memory (reviewed in McEwen and Sapolsky 1995) and dysregulation of their signalling has been implicated in a cascade of damage to regions of the central nervous system, particularly the ageing hippocampus (Sapolsky et al. 1988).

In recent years, local steroid metabolism in specific tissues by the 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes is emerging as an important control for the actions of glucocorticoids. Of particular interest to this project is the type-1 (11β-HSD1) enzyme, which was previously shown to act as a reductase in intact hippocampal cells, re-activating the relatively inert 11-ketoglucocorticoids (Rajan et al. 1996). This study also showed that carbenoxelone (an inhibitor of 11β-HSD1) attenuated kainic acid-induced neurotoxicity (which is glucocorticoid-potentiated) in the presence of 11-dehydrocorticosterone. Thus 11β-HSD1 inhibition attenuated glucocorticoid-associated neurotoxicity. Further to this, a transgenic mouse was developed, which did not express the 11β-HSD1 (Kotelevtsev et al. 1997). When the 11β-HSD1 knock-out mice were previously tested in the watermaze (a test of hippocampus-dependent memory) it was found that absence of the enzyme gave some protection from age-related impairments (Yau et al. 2001).

The aims of the current project were: to explore molecular aspects of glucocorticoid signalling which may have contributed to the protection from cognitive impairement seen in ageing 11β-HSD1 knock-out 129/Ola mice; to explore further the behavioural and cognitive implications of 11β-HSD1 absence in transgenic mice (back-crossed onto the C57BL/6J genetic background) as a function of ageing; and to establish the direction of enzyme action (oxidation or reduction) of 11β-HSD1 in other behaviourally-significant cell types of the central nervous system.

Chapter 1

General Introduction

1.1. Glucocorticoids and the HPA Axis

The world is a complex and chaotic place to be. In order to maintain our internal systems whilst interacting with multi-faceted environmental conditions, organisms have had to develop intricate responses. Some of these responses are acute and some longer term, even permanent. Glucocorticoids are involved (essential) in a wealth of these responses.

Glucocorticoids are steroid hormones secreted by cells of the adrenal cortex in response to hormonal and neural stimuli. In response to an acute stressor, an animal must rapidly re-assign metabolic resources and focus cognitive processes, such as attention and memory. The animal's response is determined by its own internal state and by the nature of the stressor. The cognitive aspects of this will be discussed in more detail (refer section 1.1.), but more somatic targets of glucocorticoids are the immune system (raised levels of glucocorticoid reducing inflammation in a flight situation) and the energy system (mobilising fat and carbohydrate resources from adipose tissue and the liver, thus maximising glucose availability). The hormonal stimulation is part of one of the classic feedback systems of biology, the hypothalamic pituitary adrenal axis (reviewed in Miller and Tyrrell 1995).

1.1.1. Modes of Glucocorticoid Action

As cortisol in humans and corticosterone in rats and mice (Figure 1-1), glucocorticoids are lipophilic and thus penetrate plasma membranes readily. They have several, identified modes of action, some of which have only recently come to light.

Cytosolic receptors

Activity depends upon activation of two cytosolic receptors, the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). MR was the first to be identified and thus is sometimes called the type I receptor and GR the type II receptor (McEwen et al. 1968; Reul and de Kloet 1985). The receptors share some properties but differ in many others, notably in their distribution and affinity for corticosteroids.

GR and MR have similar functional structures. They share 76% sequence homology in their DNA-binding domains and 59% in their hormone-binding domains (van Steensel et al. 1996). Upon binding, the receptors translocate to the cytosol, stabilised by chaperones, until appropriate ligand binding. The receptors then translocate to the nucleus (Pariante et al. 1997) and form clusters (van Steensel et al. 1996). The classical mode of action is as a ligand-activated transcription factor. The activated receptor has to dimerize (Tsai et al. 1988) and only when this has occurred can the DNA-binding domain bind to glucocorticoid-response-elements (GRE) on the DNA. The GRE's are found in regulatory regions of target genes, such as the corticotrophin releasing hormone gene (Malkoski and Dorin 1999). GR has been shown to be a more potent transcription factor than MR in cell culture experiments (reviewed in (de Kloet et al. 2000). The receptors tend to form homo-dimers, but hetero-dimers with very different effects have been described (Liu et al. 1995; Trapp et al. 1999; Ou et al. 2001b; Calle et al. 2003). In support of the possibility of hetero-dimerisation, some of the nuclear clusters have been reported to contain both receptor types in co-expressing cells (van Steensel et al. 1996).

GR also bind to negative (n) GREs which inhibit transcription by various mechanisms (Zhang et al. 1997; Drouin et al. 1993; Sakai et al. 1988). cGREs (composite) may operate where GR binds the DNA close to another transcription factor. In this manner, at a proliferin promoter, GR interacts positively with proximal activated protein-1 (AP-1) transcription factor composed of c-jun/c-jun homo-dimer, but negatively with a c-jun/c-fos hetero-dimer (Miner and Yamamoto 1992).

In order to refine our understanding of the mechanisms involved, a GR transgenic mouse ($GR^{dim/dim}$) was created, in which the GR could not dimerize and the GRE-mediated effects were lost (Reichardt et al. 1998). However, another mechanism of action was preserved. Activated monomeric GR can directly interact with protein transcription factors such as AP-1 and nuclear factor-$_{\kappa}$B. The process does not require GREs, which has made our identification of cellular glucocorticoid targets more elusive than previously thought. No such mechanisms have been shown for MR. However, effects too rapid for a translation-mediated mechanism have suggested that non-genomic effects of MR may also occur (Smythe et al. 1997) in the rodent. Interestingly, membrane bound receptors which bind corticosterone (but not dexamethasone) have been described in amphibians (Rose et al. 1993). More recently, research in amphibians has revealed corticosterone binding sites on a membrane κ opioid-like receptor (Evans et al. 2000), with rapid, non-genomic actions. It has been proposed that glucocorticoids will bind directly onto GABA receptors (Puia et al. 1994) in humans.

Thus glucocorticoids (bound to their dimerised, cytosolic receptors) can act directly as transcription factors (promoting or inhibiting transcription). In an independent mechanism, they can also directly interact with other proteins in the cell.

Receptor distribution

The receptors differ in their distribution (Reul and de Kloet 1986). GR are expressed throughout all tissues. Within the brain GR are particularly highly expressed in the hippocampus , the amygdala, septum, cingulate gyrus, the paraventricular nucleus (PVN), the cerebellum , the supra-optic nucleus and in brain stem, mono-aminergic ascending neurons (Sousa et al. 1989). The significance of this expression for emotion and memory will be discussed later. GR expression has been shown in both neurons and glia. The distribution of the MR is more restricted. Outside the central nervous system, MR is primarily a target for aldosterone. As such, it is found in high levels in the kidney distal tubules. Within the central nervous system it is found in areas associated with salt regulation (anterior hypothalamus and choroid plexus); and is highly expressed in the hippocampus (particularly CA2), the amygdala (medial and central), the olfactory nucleus, layer II of the cortex and in sensory and motor neurons of the brain- stem (McEwen et al. 1968; Brinton and McEwen 1987).

Receptor ligands

As discussed earlier, both receptor types bind physiological glucocorticoids with high affinity. However, the affinity of MR ($K_d \approx 0.5$nM) is ten-fold higher than that of the GR (Reul and de Kloet 1985). This has implications for receptor occupancy (Trapp et al. 1999) over the physiological range of glucocorticoid levels. Of interest

to this thesis, is that both receptors bind the 11-dehydrocorticosterone with such low affinity that it can be considered inert. As implied by its title, MR also binds the mineralocorticoid 'aldosterone' (Figure 1-1) with high affinity. Selectivity for glucocorticoid or mineralocorticoid ligand is conferred by a pre-receptor enzymic mechanism (refer to section 1.3.).

There are a range of agonists and antagonists used by researchers, but a GR ligand of common choice is the synthetic glucocorticoid dexamethasone, which does not occur naturally in humans, rats or mice.

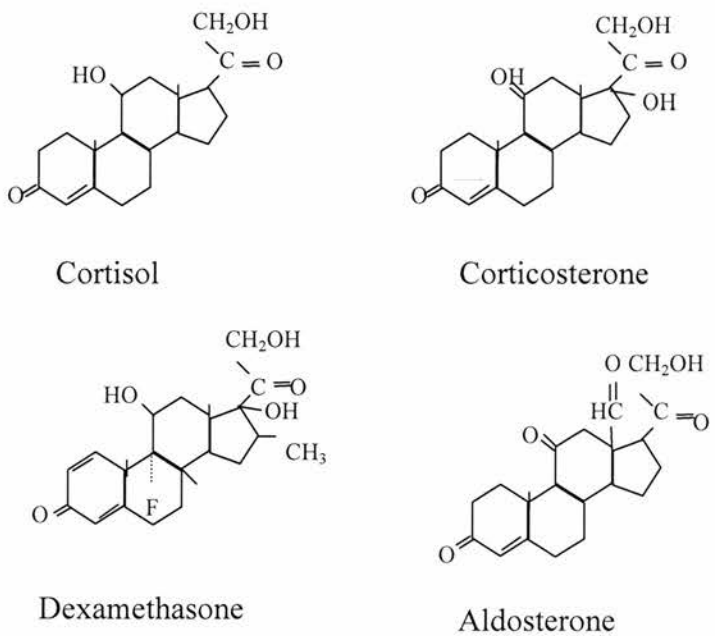

Figure 1-1: Ligands of the glucocorticoid and mineralocorticoid receptors

Cortisol (humans) and corticosterone (rats and mice) are the natural ligands for GR and MR. Dexamethasone is a synthetic GR ligand and aldosterone acts as a natural MR ligand.

It has been proposed that control of GR activity is largely determined by ligand levels and the number of receptors available, whereas MR receptor potential is modulated directly by factors such as oestrogen and progesterone (reviewed in de Kloet et al. 2000) (since MR is largely occupied under basal conditions). However, research from the Max Planck Institute suggested that levels of MR are changed rapidly and functionally by stress (Reul et al. 2000). Thus the level of expression of MR may have functional significance.

1.1.2. The HPA Axis

The concentration of glucocorticoid available to interact with receptors is controlled at many levels. The simplest to observe has been the rate of production. At times of stress, corticosterone is released from the adrenal cortex under the subtle and plastic control of the HPA axis (Figure 1-2).

Control of the HPA axis

Under basal conditions, glucocorticoid release is stimulated by adrenocorticotropic hormone (ACTH) which is released into the blood from cells of the anterior pituitary (O'Riordan et al. 1988). The release of ACTH is stimulated by corticotropin-releasing hormone (CRH-41) which has been released into the external zone of the median eminence from axons of the paraventricular nucleus of the hypothalamus. These pulsatile releases follow a diurnal pattern, hence low stimulation occurs in the sleeping phase and high in the waking. This introduction will concentrate upon the diurnal pattern of nocturnal rodents, such that low glucocorticoid levels occur in the AM and high in the PM. When glucocorticoid levels are low, it is assumed that only

the sensitive MR are occupied and as levels rise, progressively more of the GR is occupied. The pattern of occupation (level and combination of MR, GR) determines the effect (Spencer et al. 1990). During a stress response, arginine vasopressin (AVP) is also released from the hypothalamus and acts synergistically with CRH as a corticotropin releasing factor, increasing the release of ACTH (Antoni 1986).

It can be seen that such a system is open to multiple levels of control and influence. The subtleties and extent of this influence are now coming to light. The release of CRH is influenced by other areas of the central nervous system and by peripheral signals of somatic stress such as interleukin-1β (Sapolsky et al. 1987). Indirect control by the suprachiasmatic nucleus (SCN) determines the diurnal rhythm of the HPA axis (Cascio et al. 1987). In addition, the system responds to environmental requirements via influence by the limbic system (e.g. hippocampus, amygdala and subiculum) and the frontal cortex (Herman et al. 1995; Feldman et al. 1994; van de Kar et al. 1991) . These influences are both stimulatory and inhibitory; indeed feedback is a key aspect of the system.

Glucocorticoids feedback (directly) upon both CRH and ACTH. This is necessary because the effects of glucocorticoids upon the body are potent and it is essential to return levels to basal when the stress is resolved. It may also be necessary to reset the system to adjust to new sets of circumstances. In the face of an appropriate stress signal, blood glucocorticoid levels are raised. GR activation in the PVN reduces the release of CRH (Sapolsky et al. 1986b; Bradbury et al. 1991) and of AVP (Kim et al. 2001) thus negating the feed-forward drive. In addition GR activation in the anterior

pituitary reduces the expression of ACTH (Dallman et al. 1987; Shaul Feldman et al. 2002).

CRH is subject to several points of control by glucocorticoids. The number of expressing cells and the CRH production of the parvo-cellular neurons of the PVN have been shown to be GR-modulated (Dijkstra et al. 1998). It has also recently been shown in adrenalectomised rats that glucocorticoids decrease CRH mRNA stability, thus increasing its clearance (Ma et al. 2001). At the pituitary, CRH-receptors (type I) (CRH-R1) levels (necessary for the induction of ACTH expression) are subject to negative glucocorticoid control (Nikodemova et al. 2002). Yet another level of control is achieved through regulation of a CRH-binding protein (Burrows et al. 1998; Seasholtz et al. 2002). ACTH levels are controlled downstream of these changes, in addition to the, previously mentioned, local control by GR. In addition, sensitivity of the adrenal cortex to ACTH is positively regulated by the thoracic splanchnic nerve (Ulrich-Lai and Engeland 2002).

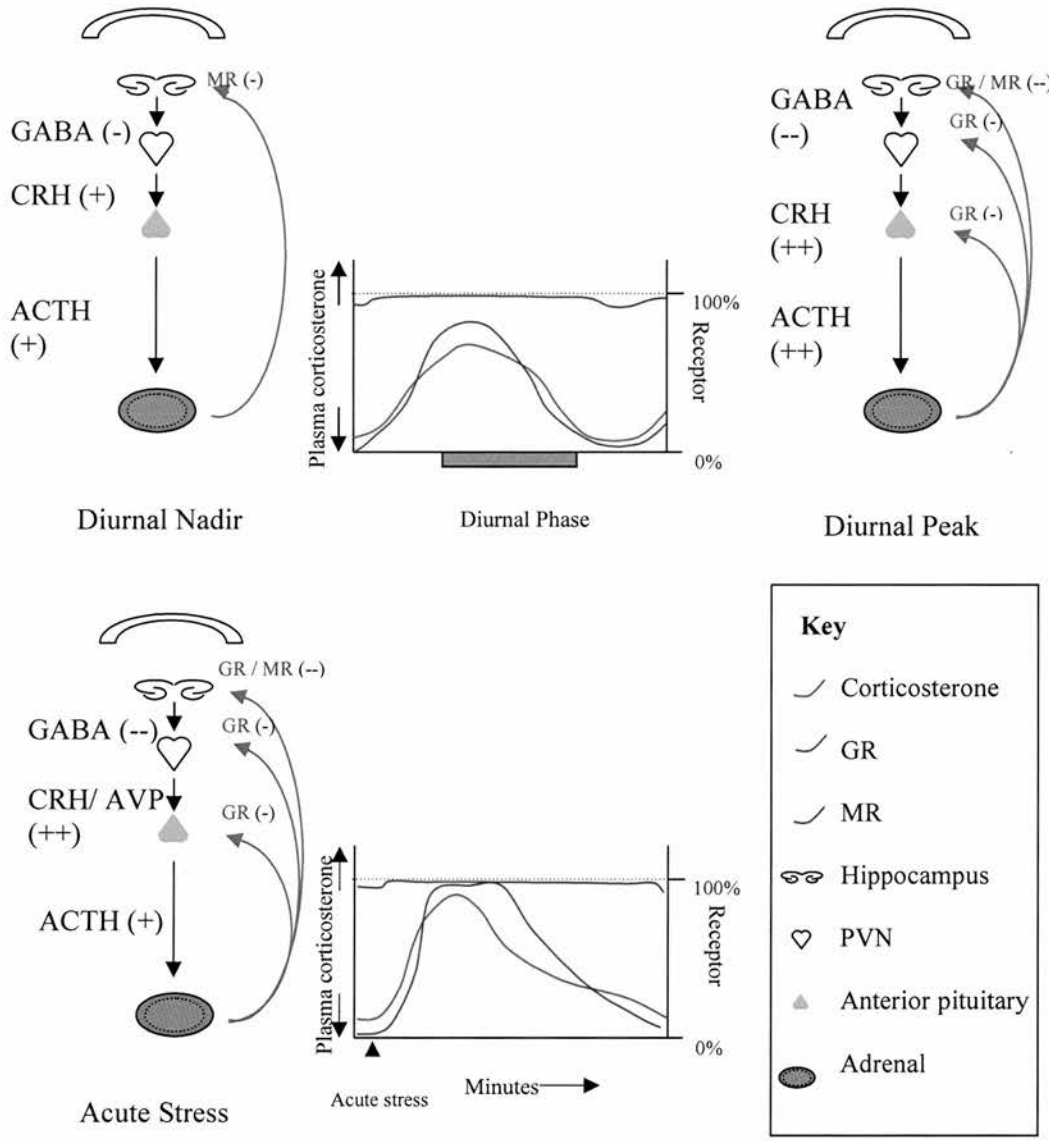

Figure 1-2: Key aspects of HPA axis control by glucocorticoids, in basal and acute stress conditions

MR is thought to be primarily responsible for tonic control of the HPA axis during the diurnal nadir. Glucocorticoid plasma levels rise in the dark phase and in response to a stressor. High levels in the diurnal peak stimulate GR and facilitate feedback upon the axis at various levels.

During a response to a stressor, increased GR occupation is thought to be responsible for feedback upon the axis and stimulation by AVP. ('-' represents an inhibition; '+' represents a stimulation)

The Hippocampus and the HPA axis

At this point the hippocampus should be drawn into discussion. The hippocampal formation (the structure of which will be discussed later) has high levels of GR and MR. It exerts negative influence upon the PVN through the fimbria-fornix (fibre bundle). Lesion of this pathway leads to increased HPA activity (Sapolsky 1991). It is now understood that glutamatergic neurons from the ventral subiculum synapse GABA-ergic neurons of the bed nucleus of the stria terminalis (BST) (Herman et al. 1995). The BST then transmits information to the peri-PVN zone and the PVN (Cullinan 1993). In so doing the BST exerts negative control over the PVN during psychological stressors and is able to relay hippocampal signal to the PVN.

The general consensus is that MR activation within the hippocampus is involved in maintenance of basal nadir corticosterone levels, but has a permissive effect upon GR effects (Spencer et al. 1998). The lower affinity GR is progressively activated at higher levels of HPA activation. A role for the hippocampus in mediating glucocorticoid control of the HPA response to stress was initially proposed in ageing research (Sapolsky et al. 1984). Latterly, more targeted research has established a role for hippocampal GR in the stress response, with centrally administered GR antagonist resulting in activation of the HPA (Feldman and Weidenfeld 1999). Use of a GR antagonist had no effect upon nadir levels of activation but did decrease inhibition of the HPA in the PM phase and after acute stress (Spencer et al. 1998).

1.1.3. Factors Affecting the Access of Glucocorticoids to their Receptors

It has been shown that glucocorticoid effects can be modulated at the level of glucocorticoid production, at the receptor and downstream of receptor activation. In addition, the levels of glucocorticoid which reach the receptors can be regulated at intermediate points.

Binding protein

A major player in this regulation is corticosterone binding globulin (CBG). This protein is produced in the liver and selectively binds corticosterone with relatively high affinity. Low levels of plasma steroids are also bound non-selectively to albumin with low affinity (Dunn et al. 1981). However, binding to CBG has a significant impact upon the availability of corticosterone. At the diurnal nadir, 95% of the corticosterone can be bound. It is of interest that 11-dehydrocorticosterone binds with a much lower affinity and is essentially free in the plasma (reviewed in Seckl and Walker 2001). Thus at the nadir, effective levels are similar to those of "free" corticosterone. Another point of interest is that some CBG (although not able to cross the blood-brain-barrier) is found within cells of the pituitary and contributes in modulating access of corticosterone to local GR (Tannenbaum et al. 1997). CBG has been shown to be decreased after stress (Fleshner et al. 1995) and is GR-down-regulated (Cole et al. 1999). CBG is increased in the PM phase in rodents (Meaney et al. 1992), controlling the amount of "free" glucocorticoid.

A glucocorticoid pump?

The lipophilic structure of corticosterone enables easy access through the blood-brain-barrier and into cells. However, it has been shown that dexamethasone is 'pumped' out again by the multi drug resistant protein MDR-1 (Webster and Carlstedt-Duke 2002). Thus, it may be argued that peripheral treatment with dexamethasone (under the conditions used) may only be effective at the pituitary. The same study suggested that endogenous glucocorticoid may also be subject to such clearance by the same MDR-1 and/or by a multidrug resistant related protein MRP-1 and indeed a more recent study has demonstrated a related protein which does indeed export cortisol (Pariante et al. 2003).

Metabolic conversion

Corticosterone is also subject to metabolic clearance from the plasma. The details of this process are outwith this thesis, but it should be appreciated that a series of enzymes influence the half-life of corticosterone and therefore the longevity of changes in concentration.

The final aspect of pre-receptor control to be introduced is that of the 11β-hydroxysteroid dehydrogenases. 11β-hydroxysteroid dehydrogenase type-1 and type-2 are enzymes involved in the bi-directional conversion of 11-keto glucocorticoids (with glucocorticoid activity) and 11-dehydroglucocorticoids (without glucocorticoid activity). They play key roles in determining the peripheral levels of glucocorticoid and intracellular levels in expressing cells. As a focus of this thesis they deserve individual discussion (refer to section 1.3).

1.1.4. Summary

Glucocorticoids exert their wide influences through their cytosolic type-I (MR) and type-II (GR) receptors. The result of receptor activation depends upon the level of ligand gaining access to receptor, the ratio of GR to MR receptors and factors downstream of receptor activation. Glucocorticoid release from the adrenals is under neuronal and hormonal regulation. The hormonal regulation by the HPA axis has both negative and positive feedback aspects. The HPA axis is also sensitive to influence from other areas of the brain, the hippocampus being of particular interest in the present thesis.

1.2. Glucocorticoids and Cognition

There is a vast history of data establishing the two-way relationship between glucocorticoids and cognition. This data has come from animal and clinical studies, exploring links with anxiety, depression, memory, attention and motor performance. As discussed previously, glucocorticoid receptors are found in brain regions which have been linked with these functions, such as the amygdala, the hippocampus, the brain stem and the cerebellum.

1.2.1. The Structure of the Hippocampus

The hippocampus is a highly organised structure situated in the medial-temporal lobe, forming part of the limbic system (Figure 1-3) (Johnston and Amaral 1998; Kandel 2000). Its structure and susceptibility to seizure made it an early source of interest, with the structure and basic connections being largely described by Ramon y Cajal and Lorente de No in the early 20th century. The hippocampal formation consists of the hippocampus proper, the dentate gyrus, subiculum and entorhinal cortex. The hippocampus receives higher input through the entorhinal cortex and the fornix-fimbra (see below).

The entorhinal cortex projects to the dentate gyrus (DG) and the cornu-ammonis (CA3 and CA1) in a structured manner. This glutamatergic input is termed the 'perforant path' and enters the hippocampus proper through the hippocampal fissure. The dense layer of granular neurons give rise to unmyelinated, generally glutamatergic and highly branched 'mossy fibers', which terminate on proximal dendrites of the pyramidal neurons of CA3. Each mossy fiber can connect with up to

14 pyramidal neurons and each pyramidal neuron may receive input from 50 granular neurons. Thus the influence of a granular neuron may be vast. In addition, the CA3 pyramidal neurons project to their contra-lateral counterparts (e.g. left to right hippocampus) via the commissural pathway. The CA3 also project to the pyramidal neurons of the CA1, via the 'Schaeffer collaterals'. From there the CA1 and the subiculum project to the deep entorhinal cortex which then returns input to other areas. The CA3 also projects via the fornix-fimbra, an important trans-synaptic connection with the hypothalamus. It is important to note that all of these connections are influenced by inter-neurons throughout the molecular layers. In addition the polymorphic layer and the granular neurons of the dentate gyrus, the pyramidal neurons of CA1 and CA3 receive important noradrenergic input from the locus coeruleus and serotonergic input from the raphe nuclei.

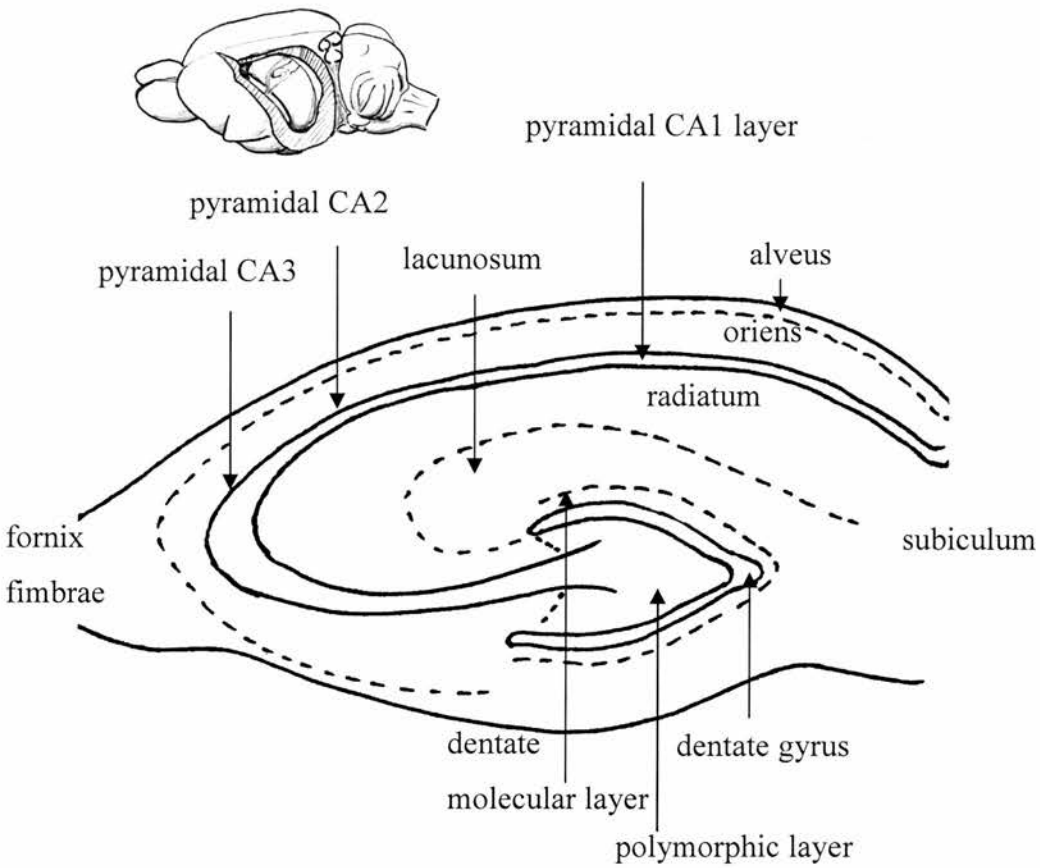

Figure 1-3: The gross structure of the rodent hippocampus

The dense cellular layers of pyramidal neurons and the dentate gyrus are connected by several layers of more sparce glia, interneurons and their connections. The perforant path enters at the hippocampal fissure and terminates on the dentate gyrus (DG) and the cornu ammonis regions (CA1 and CA3). The DG projects to the CA3, via the highly collateralised mossy fibers, and the CA3 projects out of the hippocampus through the fornix and through the Schaeffer collaterals to the CA1. This in turn projects out of the hippocampus proper and into the subiculum.

1.2.2. Hippocampal Memory

The name HM is engraved in neuroscience history. This young man suffered such debilitating epilepsy that in the early 1950s he underwent surgery, which removed much of his hippocampus and overlying cortex. He was thenceforth greatly impaired in his ability to form 'explicit' memories, though retained pre-surgery memories and was able to create new 'implicit' memories (reviewed in Deweer et al. 2001).

'Explicit' memories are recognised as those to which humans have verbal access, the 'where, when and who' memories. They are largely coincident with 'declarative' memories, based upon another model. 'Implicit' memories are those to which we do not have direct verbal access. Who can explain exactly how they judge catching a ball? A later well-documented case 'RG' was, at post-mortem, found to have suffered selective damage of the CA1 and in life was unable to form new memories.

The hippocampus has now emerged as an intermediary in memory formation, making associations between internal and external cues, whilst maintaining the individuality of the cues. Much confusion over the nature of the memory storage has been rooted in collateral damage to the entorhinal cortex and subiculum during lesioning and in the type of memory models tested. It is essential to consider the latter when attempting to model, human explicit amnesia. The idea of modelling 'declarative' memory in animals often generates disquiet. There are several lines of evidence which suggest that the ability to 'declare' is a bi-product of hippocampal memory rather than an essential element (reviewed in(Eichenbaum 1999)).

Spatial memory in the hippocampus

Insight into the associative function of the hippocampus was gained when 'place cells' were identified in the cornu-ammonis of the rat (Speakman and O'Keefe 1990). These were cells which 'fired' when the rat was presented with a particular set of spatial cues (i.e. was in a particular section of a cued maze). This 'spatial memory' in the rat had been under investigation by this group for some years (O'Keefe 1979) (O'Keefe and Speakman 1987). A particularly useful tool for investigating spatial memory, was developed by Richard Morris of Edinburgh (Morris 1984). This experimental paradigm (described in more detail in Chapter 3) measures an animal's ability to learn associations between positional, visual cues in a room and an escape platform in a pool of water.

Non-spatial memory in the hippocampus

Hippocampal learning is not restricted to spatial cues. The hippocampus has been shown to be involved in conditional learning where a plastic association has to be made between cue and context (Sutherland et al. 1989) and in non-conditional learning (Bunsey and Eichenbaum 1996). One study has associated different areas of the dorsal hippocampus with spatial and non-spatial memories (Hampson et al. 1999).

Mechanisms of memory storage in the hippocampus

There is much debate over the mechanism of storage. As place maps can last for several days but can be modified, there would have to be stability and plasticity involved in storage. A popular cellular candidate has been long-term-potentiation

(LTP) (Bannerman et al. 1995) in the Schaeffer collateral inervation of the CA1 pyramidal neurons. Such potentiation of the synapse's ability to transmit a signal can develop within minutes and last for several hours in its early phase. Accumulated signalling at more than one synapse within this phase, can lead to a more stable late-phase which can last for days (reviewed in Malenka 1994; Kandel 2000). Such potentiation is stable and associative, reminiscent of hippocampal-dependent memory. LTP was first outlined by Bliss and has received much attention and research as a candidate mechanism for memory formation (Bliss and Collingridge 1993). There has been controversy regarding the role for LTP in long-term hippocampal memory (Sutherland et al. 1993; Saucier and Cain 1995; Nosten-Bertrand et al. 1996). Thus evidence about the effects of glucocorticoids upon LTP should not be assumed to play a role in long-term memory without direct evidence.

The early phase involves high frequency glutamate signalling of non-NMDA receptors, which leads to depolarisation of the membrane and eventually release of a blocking magnesium from NMDA receptors (Herron et al. 1986). This stimulation can be induced with theta (3-13Hz) electrical stimulation of the presynaptic neuron (Bliss and Gardener-Medwin 1993). The subsequent influx of calcium triggers a signalling cascade which phosphorylates and potentiates the sensitivity of the non-NMDA receptors, recruiting more to the signalling in the process (Malenka et al. 1989; Frank et al. 1989). A retrograde messenger then enhances transmitter release from the presynaptic terminal. If the calcium signal is maintained, an adenylyl cyclase is activated and protein synthesis initiated which leads to structural changes such as the placement of new receptors in the postsynaptic membrane. Presynaptic

changes are also known to occur, which increase the likelihood of transmitter release. Impaired LTP in a transgenic model has been shown to lead to unrefined place maps, but their formation is not dependent upon this LTP (Tsien et al. 1996).

There is also a non-associative form of LTP which occurs in the mossy-fiber pathway. This is initiated by an influx of calcium (induced by titanic stimulation) in the pre-synaptic neuron and can be modulated through β-adrenergic receptors (Fisher and Johnston 1990). Interestingly, performance in a hippocampus dependent watermaze task was correlated positively with NMDA receptors in the CA3 region in young and aged rats (Adams et al. 2001).

Another form of plasticity, long-term-depression (LTD) may be involved in responses to novelty in the CA1 region (Braunewell and Manahan-Vaughan 2001) and in conditional learning in the cerebellum (Koekkoek et al. 2003). Indeed, it may be that stress levels of glucocorticoids encourage a switching from LTP to LTD in the CA1 region (Xu et al. 1998). A later study, could not show that this switch to LTD was responsible for stress-related impairement in spatial learning (Xiong et al. 2003).

The hippocampus appears to be an intermediate storage for new and rehearsed memories. When electrical activity was measured locally in the CA1 region of rats, a relative increase in theta type 1 activity (6-9Hz) was associated with the processing of spatial memory in a watermaze task (Olvera-Cortes et al. 2004). Theta waves are also associated with signal communication with the neo-cortex and through measuring these, it is thought that long-term consolidation occurs during periods of

inactivity (sleep) and memories stored within the cortex (Buzsaki 1996; Hirase et al. 2001).

1.2.3. Glucocorticoids in the Hippocampus

As has been previously mentioned, the hippocampus has a high density of both MR and GR. Some 35 years ago, a series of *in-vivo* experiments conducted by Bruce McEwen (McEwen et al. 1968) demonstrated high retention of labelled corticosterone in the hippocampus. When compared with dexamethasone (a GR ligand), the curious binding profile revealed the presence of MR in addition to GR.

The effects of glucocorticoids upon hippocampal memory

Glucocorticoids affect hippocampal memory in a concentration and context dependent manner. Continuous, central antagonism of the MR with spironolactone reduced rat performance in the standard Morris watermaze (Yau et al. 1999). An earlier study had looked at the effects of the similarly applied GR antagonist RU38486. In this case, an improvement in performance was shown (Oitzl et al. 1998b). These studies illustrate the proposed inverted U shape relationship between hippocampus performance and glucocorticoid level. A later study in young adult humans used the natural diurnal variation in glucocorticoid as a platform for investigating this same relationship. Lupien *et al.* found low and high levels of glucocorticoid impairing and intermediate levels facilitating in a delayed recall test (Lupien et al. 2002). It is interesting that transgenic mice with disrupted GR function, displayed impaired allocentric (relating the subject to spatial context), but retained egocentric (self-motion) memory in the water maze and in a radial maze

(Steckler et al. 1999). This should be related back to the function of the hippocampus in spatial memory.

However, the story is not simply of glucocorticoid concentrations. The water-maze acts as a stressor to the rat and naturally raises glucocorticoids as a trial progresses. When the stress of the swim is increased (by decreasing the water temperature), with a subsequent increase in plasma corticosterone, memory is improved (Sandi et al. 1997). In addition, when adrenalectomised rats are given comparable levels of exogenous corticosterone directly after water-maze training, their performance is improved (Sandi et al. 1997). This suggests the importance of an acute increase in glucocorticoid associated with the swim. The context of the stressor and/or rise in glucocorticoids have been shown to be important. It has been shown that exposure to a cat stressor after acquisition in a complex watermaze test can impair performance in rats (Diamond et al. 1999). Thus rises in glucocorticoid which are within the context of learning (and are not excessively high) may reinforce learning, whereas glucocorticoid increases out of context may inhibit learning. There are, of course, other potential mediators of stress responses which may be involved. Novelty or exposure to a psychological stress can rapidly modulate acetylcholine, glutamate, noradrenaline and serotonin activity in the hippocampus (reviewed in Habib et al. 2001).

However, chronic glucocorticoid treatment would impair performance (a phasic treatment with GR antagonist was shown to impair rather than improve water maze performance (Oitzl et al. 1998a). Thus timing of the increase is important.

It appears that MR and GR do not simply have accumulative effects within the hippocampus. MR may be involved in search strategies and GR in consolidation (Oitzl and de Kloet 1992). This would certainly fit with the temporal pattern of low glucocorticoid levels as the animal establishes its search pattern and higher levels as it completes the trial and has to consolidate information. It should also be noted that GR and MR have some distinct GRE targets. Thus, a change in the balance of activation of these receptors will result in the activation of different genes.

There is very good evidence that acute glucocorticoid effects upon hippocampal memory consolidation are mediated through effects upon the basal-lateral-amygdala (BLA) and nucleus accumbens (NAc) pathway (Roozendaal et al. 2001; reviewed in Roozendaal 2000). All three sites of this interaction are glucocorticoid sensitive.

Glucocorticoids can affect excitability and energy metabolism in the hippocampus within minutes (Horner et al. 1990) and precipitate structural changes within days (Woolley et al. 1990). Effects tend to be subtle until the cell becomes excited. Acute stress has been shown to inhibit potentiation in the hippocampus (Foy et al. 1987) while unreplaced adrenalectomy reduced the threshold for primed burst potentiation (a form of LTP) (Diamond et al. 1989). Further work showed glucocorticoids exerting an inverted U relationship with primed burst potentiation in the hippocampus (Diamond et al. 1992). Notably, in the DG, CA1 and CA3 (commissural-associational input), MR increased and prolonged LTP, whereas GR suppressed LTP and produced LTD (Pavlides and McEwen 1999) and 1993). The mossy fiber CA3 input (NMDA receptor independent) was not affected. In fact, the general picture of CA1 output is that it is maximised with MR activation and

progressively reduced with GR (reviewed in De Kloet et al. 1998). A recent study supported a role for the balance between GR and MR in mediating context-dependent influence upon LTP (Korz and Frey 2003). Glucocorticoids may also affect memory through modulation of neurotransmitter-mediated excitability (reviewed in Joels et al. 1995).

The effects of glucocorticoids upon hippocampal structural integrity

Chronic stress has been shown to have varied effects upon the hippocampus. Several studies have found smaller hippocampal volumes associated with high corticosterone or stress (Bremner 1999; Sheline et al. 1996). In some studies, reduced hippocampal volume has been correlated with cognitive deficit (Kaye et al. 1997; Sykova et al. 2002). These reduced volumes have been ascribed to cellular atrophy, indeed cell loss has been found in the CA3 region (Sapolsky et al. 1986a). In a study which challenged a role for physiological glucocorticoid in hippocampal, long-term high dexamethasone treatment in rats induced cell loss in the DG and CA3, whereas high levels of corticosterone or aldosterone had no effect (Sousa et al. 1999). This suggested that high levels of GR activation would damage these regions without the occupation of MR which occurs under physiological conditions. The question of cell loss may be resolved (to some extent) by the finding that mice which were MR-/- but not GR-/- showed impaired neurogenesis in the DG (Gass et al. 2000). This may have been directly a result of MR loss and/or through increased GR activation secondary to higher circulating glucocorticoid. However, other studies have challenged the neuronal loss principle (Leverenz et al. 1999) and suggested a high glucocorticoid induced loss of mossy fiber complexity (Woolley et al. 1990;

Watanabe et al. 1992; Magarinos et al. 1996). Thus glucocorticoids may act to endanger or to impoverish hippocampal neurons.

There is clear *in vitro* evidence that glucocorticoids potentiate the endangerment of pyramidal neurons by excitatory amino acids (eg. glutamate), free radicals, hypoxia and glucose depletion (reviewed in Sapolsky 1992). These mechanisms appear to be hippocampus specific, though this has not been extensively evaluated and more widespread glucocorticoid toxicity to neurons of the developing brain, notably of the cerebellum, has been detailed (Gramsbergen and Mulder 1998).

Glucocorticoids may potentiate excitatory amino acid in several ways. They increase and prolong glutamate levels at the synapse by inhibiting the reuptake by glia (Stein-Behrens and Sapolsky 1992). This may be related to inhibition of glucose utilisation (Horner et al. 1990; Virgin, Jr. et al. 1991; Freo et al. 1992). Glutamate signalling results in an increase in Ca^{2+}, which in itself may be neurotoxic and related to free radical damage of the cell. Indeed, glucocorticoids are known to inhibit some antioxidants (McIntosh et al. 1998). Of course, a reduction in glucose may also lead to increased free radicals. High levels of glucocorticoids have been shown to inhibit the activity of several neuronal growth factors. Notable, is the reduction of brain-derived neurotrophic factor in the CA3 and DG (Hansson et al. 2000; Hansson et al. 2003). Brain-derived neurotrophic factor has been associated with LTP as well as the complexity of dendrites (Yamada et al. 2002) and has shown to be reduced in Alzheimer's disease and the ageing primate brain (Hayashi et al. 2001).

It is likely that some of these changes are not permanent. Patients with Cushing's disease (chronically high glucocorticoids) were treated to reduce their plasma

cortisol with a subsequent increase in their hippocampal volume (Starkman et al. 1999). Changes have also been achieved with antidepressant treatment (refer to section 1.2.6.).

It is not a simple case of glucocorticoids being the root of all evil for the ageing brain. It is now well accepted that a balance of glucocorticoid is essential to the brain, as the granular neurons of the DG begin to undergo apoptosis after only a few days of unreplaced adrenalectomy. This process is prevented by MR activation, but not GR (Bye and Nichols 1998). Thus glucocorticoids modulate the excitability of neurons and, more chronically, influence the cellular or dendritic integrity and complexity of the hippocampus.

1.2.4. The Structure of the Cerebellum

The cerebellum, like the hippocampus, is a layered and highly organised structure and is the second structural point of interest in this thesis. It has traditionally been associated with motor co-ordination and, more recently, with classical conditioning. The structure has been associated with autism (Kern 2003) and dyslexia (Nicolson et al. 2002), demonstrating possible new roles for the cerebellum. One reason for interest for this thesis is the high cerebellar expression of both GR (Sousa et al. 1989) and 11β-HSD1 mRNA (Lakshmi et al. 1991).

The cerebellum cortex has an extensive, highly folded structure (Figure 1-4) (Ghez 1991; Llinas and Walton 1998). It consists of an outer molecular layer, a monolayer

of large Purkinje neurons which is closely associated with a dense granular layer. Below this sits a layer of white matter.

There are two major inputs to the cerebellum cortex. The 'climbing fibres' are the highly collateralised axons of the inferior olivery nucleus, which receives input from the spinal cord, brain stem, the motor cortex and cerebellum nuclei. Each Purkinje neuron receives one climbing fibre. The 'mossy fibers'come from many areas, including the cortex (through the pontocerebellar pathway), the cerebellar nuclei, the spinal cord and the vestibular nuclei. They enter through the cerebellar peduncles, go to the deep cerebellar nuclei, branch into the white matter and terminate on the granule cells layer. The granule cells then project into the molecular layer. There may be synapses formed with Purkinje neurons at this point, but in the main the axon splits to form a 'T' and parallel fibers which synapse with a vast number of Purkinje neurons and other neurons of the molecular layer. Both climbing fiber and parallel fiber activation are suppressed by the GABAergic Basket cells etc, such that parallel fibre activation results in transient activation and climbing fiber in slightly longer activation. Because of indirect inhibition of the mossy fiber synapses, climbing fiber activation is dominant.

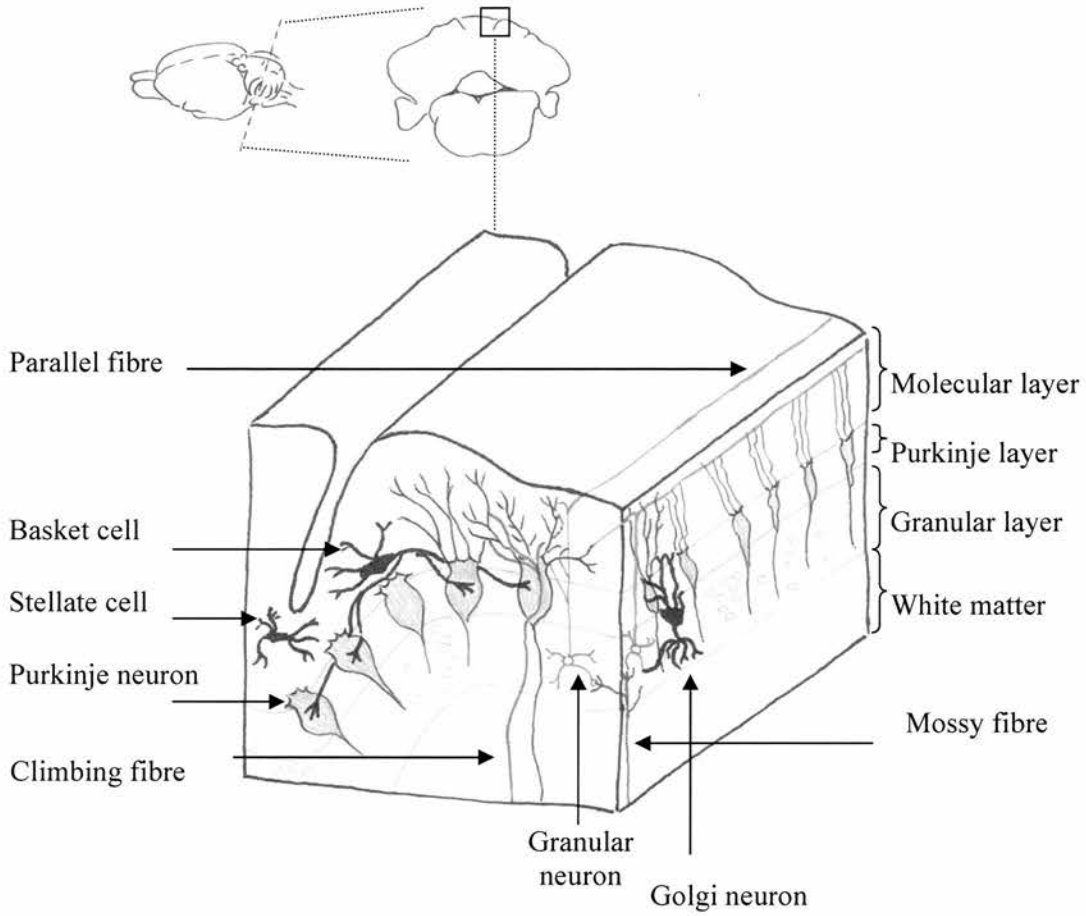

Figure 1-4: The structure of the rat cerebellum

A cartoon of the principle cortical structures of the cerebellum (Ghez 1991). The primary input comes from the mossy fibres and the climbing fibres. The mossy fibres synapse onto the granular neurons and the climbing fibres synapse directly onto the Purkinje neurons. The granular neurons may synapse directly onto a Purkinje neuron or onto distal Purkinje neurons via the parallel fibres, exerting a stimulatory, glutamatergic influence. Basket cells and Golgi neurons exert a inhibitory, GABAergic influence. The main output from the cerebellum comes from the Purkinje neurons.

1.2.5. The Cerebellum and Learning

A role for the cerebellum in learning is now well established. The structure is known to play a significant role in the classical conditioning paradigm of the 'eye-blink test' (Mauk and Donegan 1999). More predictably, a cerebellum role has been established in motor learning (Thach 1998). Despite the relatively high levels of GR found in the cerebellum, the published research into glucocorticoid effects upon cerebellar learning is sparse. A recent notable exception is a vestibular compensation mechanism which involved the paraflocculus (Yamanaka et al. 2000). Of particular interest to this project is the new role given to the cerebellum in spatial learning. Hemi-cerebellectomy experiments have implicated the cerebellum in the planning stages of spatial learning (Petrosini et al. 1996) and a transgenic mouse with Purkinje cell degeneration was able to use proximal but not distal cues (Belzung et al. 2001). Loss of the granular layer integrity meant that the mice could not perform the visible version of the maze.

If there is degeneration of the cerebellum with ageing, then the previously discussed results will impact upon the interpretation of watermaze results. Indeed, age-related loss of volume has been identified in patients. It has been assumed that this loss has been in the Purkinje layer, but recent stereological analysis of the human brain suggests that this loss of volume is in the white matter layer and in the volumes of the Purkinje neuron cell bodies rather than their number (Andersen et al. 2003).

1.2.6. Glucocorticoid Receptor Plasticity

The glucocorticoid receptors and their message are subject to some plasticity. GR transcription has been shown to be auto-down regulated *in-vitro* (Rosewicz et al. 1988). In accordance with this, GR mRNA was shown to be decreased in the CA3 of rats treated with high dexamethasone (Herman et al. 1989). In the same study, adrenalectomy increased GR mRNA in the CA1 and both GR mRNA and MR mRNA in the DG; this was shown to be reversed with dexamethasone treatment (GR dependent). Although dexamethasone is normally extruded from the brain because of the presence of the multi-drug resistance transporter (MDR_{1a}) (Webster and Carlstedt-Duke 2002), the high concentration of dexamethasone used in these studies would saturate the transporter and so some dexamethasone would have remained in the brain. Interestingly the adrenalectomy induced MR mRNA in CA1 was also reversed with dexamethasone treatment, although high dexamethasone treatment in the intact animal had no effect upon MR. A negative autoregulation of GR appears counter-productive for feedback upon the HPA, potentially reducing hippocampal control over the hypothalamus. A more recent study indicated that GR mRNA in the hippocampus is regulated by GR and MR and that MR mRNA is autoregulated (Chao et al. 1998). The negative autoregulation of GR protein (after chronic ADX or chronic high glucocorticoid treatment) has been confirmed by Western analysis (Spencer et al. 2000). MR has recently been shown to be rapidly increased in the hippocampus after an acute psychological stressor (Reul et al. 2000) and was associated with increased MR-mediated inhibition of the HPA axis. The mechanism

for this increase has yet to be clarified, but the study demonstrates a plastic control of/by MR which was previously thought to be mediated by GR alone.

There has been little work looking at glucocorticoid receptor plasticity in the cerebellum. However, a study looking at long-term (over 8 days) stress in rats, showed down regulation of GR mRNA (Kitraki et al. 1999) in the both hippocampus and the cerebellum.

1.2.7. Glucocorticoids and Mood Disorders

In light of the key role given for glucocorticoids in managing the daily responses to stress, it should not be surprising that imbalance in the system has been associated with depression and anxiety. Patients with major depression show an increased frequency of CRH and ACTH pulses (Rubin et al. 1987) and depressed patients with cognitive impairment show increased 24 hour urine output of cortisol metabolites (Rubinow et al. 1984). During depressive episodes they often show elevated cortisol levels and flattened diurnal rhythms (Cervantes et al. 2001). Some studies have linked endogenous (as opposed to reactive / exogenous) depression with increased 24 hour glucocorticoid output (Thase et al. 1996; Young et al. 2001).

A possible diagnostic tool, the dexamethasone suppression test has been developed. Patients with a range of mood disorders showed increased plasma cortisol levels, suggesting impaired feedback (Carroll 1982). This test has been largely superseded by the dexamethasone/CRH test (Deuschle et al. 1998). The test involves a pre-treatment with dexamethasone, followed by a treatment with CRH. The dexamethasone probably suppresses the ACTH production at the pituitary,

decreasing adrenal production. However, the central aspects of the axis then only detect the decreased cortisol (the dexamethasone not penetrating) and reduce their inhibition. The drive and adrenal production is increased. The treatment with CRH should reveal changes in feedback aspects of the supra-hypothalamic sites (although it could also reveal differences in sensitivity to CRH). In depressed patients the subsequent CRH production is amplified and the sensitivity of the system to dexamethasone is reduced, suggesting an impairment of the GR feedback. There is also suggestion of impairment of the AVP aspects of the system in major depression (Scott and Dinan 2002; Wigger et al. 2003). In the Munich Vulnerability Study, the dexamethasone / CRH test has been used to identify the relatives of depressed patients who may be at risk of developing problems (reviewed in Muller et al. 2002). Such results do of course raise the issue of a predisposing genetic factor, which is largely not explained by mutations in the GR.

Successful treatment with tricyclic antidepressants has been shown to normalise the HPA rhythm of depressed patients (Laakmann et al. 2003; Deuschle et al. 2003). This does beg the question of whether the rhythm is the primary problem or is secondary to neurotransmitter dysregulation. It may indeed be the case that the neurotransmitter problems are secondary to the glucocorticoid dysfunction (reviewed in (Gass et al. 2001)) but are generators of at least some symptoms. In support of this, there is evidence to suggest that antiglucocorticoid treatment may itself be antidepressant (Wolkowitz and Reus 1999).

Hippocampal MR has been related to anxiety (Smythe et al. 1997). However, a mouse with a conditional, brain specific loss of GR, showed reduced anxiety (Strohle

et al. 1998) and over-expression of GR in forebrain increased anxiety-related behaviours (Wei et al. 2004). Results from the GR $^{dim/dim}$ mouse, suggest that GR-GRE interactions are not involved in anxiety behaviour (Oitzl et al. 2001). Thus some forms of anxiety may be related to in-trans aspects of corticosteroid receptor activation, in a manner which is rapid rather than the delayed progress of DNA transcription and translation.

1.2.8. Glucocorticoids and Ageing

Old age is becoming more of an interest and concern to the general population. In general we have accepted that some memory loss and cognitive decline are aspects of old age. But the increasing affluence of western society is leading to expectations that we will be active and productive in retirement. More recently, it has become apparent that future ageing populations will have to be mentally and physically healthy enough to continue earning an income. At present, a significant proportion of the population will experience cognitive decline with ageing. Thus, decline is indeed an aspect of ageing but is not a fait accompli. Is it possible that decline can be delayed or avoided?

Frequencies of cognitive decline in aged populations, similar to those described in humans, have been described in some laboratory rodent studies (Issa et al. 1990; Levy et al. 1994; Yau et al. 1995; Luparini et al. 2000; Sykova et al. 2002). Such hippocampal –type cognitive decline is associated with dysregulation of the HPA axis in ageing men (Lupien and Meaney 1998) and critically, glucocorticoid levels appear to correlate with the variance in cognitive function with ageing, rather than

representing a constant feature of age *per se*. A possible link between hippocampal dysfunction in the aged and glucocorticoids was demonstrated in an early study of adrenalectomy and low dose corticosterone replacement in middle-aged rats, which show improved ageing in those with controlled glucocorticoid (Landfield and Eldridge 1981). However, the Brown Norway strain of rat shows facilitated cognitive function with age when glucocorticoid levels are higher in earlier life and improved markers of function relative to other strains (Gilad et al. 1987). This particularly long lived strain perhaps models successful ageing.

Evidence suggests that the hippocampal impairment in aged rats decreases their ability to adapt their 'hippocampal map' in response to a change of environment (Tanila et al. 1997). There appears to be a loss of plasticity. In addition, there has regularly been shown to be a loss of volume in the hippocampus with ageing, which goes beyond the loss found in regions such as the frontal cortex or the cerebellum (Raz et al. 2004; Kaye et al. 1997; Rodrigue and Raz 2004).

There have also been several reports of reduced MR and / or GR mRNA expression in the hippocampi of ageing rats (Sapolsky et al. 1983). Such results are, by no means, always found (Table 1-1). A theory of progressive hippocampal damage is encapsulated in the 'Glucocorticoid cascade hypothesis' developed by Sapolsky and Landfield (Landfield et al. 1978; Landfield and Eldridge 1981). An initial dysfunction (as yet, not identified) results in impaired HPA feedback which results in increased exposure of the neurons to glucocorticoids. This exposure results in neuron damage, which then impairs feedback. There then follows an accumulation of damage and increasing glucocorticoid levels. The decrease in GR may be a

neuroprotective response, which then proves to be deleterious to the system. The age-related decrease in MR protein parallels the mRNA (Hassan et al. 1999), but the reduction in GR binding sites may precede their mRNA .

Change in receptor expression or message

↓ **MR** (Rigter et al. 1984; Reul et al. 1988; Lorens et al. 1991; Hassan et al. 1999)

↓ **GR &** ↓ **MR** (Issa et al. 1990; van Eekelen et al. 1991)

↓ **GR mRNA** (Cizza et al. 1994)

↓ **MR mRNA** (Sarrieau and Mormede 1998; Hassan et al. 1999)

↓ **GR &** ↓ **MR mRNAs** (Bizon et al. 2001; Yau et al. 2002)

No change in mRNA (Yau et al. 1994b; van Eekelen et al. 1991)

Table 1-1: Published findings of adrenocorticoid receptor changes in the ageing hippocampus

↓ show reduced levels.

It should be remembered that glucocorticoids may not be the only source of age-related changes in the hippocampus. Other changes may be independent of glucocorticoid -related changes or may interact. A loss of septo-cholinergic neurons in aged rats has appeared to precede hippocampal damage (Gilad et al. 1987). The activity of this cholinergic input is increased with stress and may be another link

between stress and age-related damage. In addition, there is broad evidence that calcium dysregulation may be involved (reviewed in Verkhratsky and Toescu 1998). As an example, ageing and stress may lead to slower autophosphorylation of CAMKII at Thr305, which maximises the binding of calmodulin in the post synaptic neuron and results in higher levels of Ca^{2+} (Schulman et al. 1992).

The question remains, as to why there should be similar rates of cognitive ageing in inbred (e.g. Wistar rats) and outbred (e.g. human beings) populations. Is this a 'nature' or 'nurture' effect? Early life events can re-programme hippocampal glucocorticoid profiles. In the neonatal handling paradigm (Meaney et al. 1988), rat pups are handled daily for 15 minutes for the first 1 to 2 weeks of their lives. Acting through increased maternal attention (licking and grooming) and subsequent activation of 5-HT pathways to the hippocampus; handling results in a permanent increase in the GR density of the hippocampus and pre-frontal cortex. This sensitises the HPA axis to glucocorticoid feedback, maintaining lower glucocorticoid levels throughout life (Vallee et al. 1997). Prolonged maternal separation and prenatal stress can have the opposite effect (Plotsky and Meaney 1993; Vallee et al. 1997). There appears to be an influence of environment beyond weaning. It has been shown that environmental enrichment (long and short term) can reduce age-related cognitive deficits (Kobayashi et al. 2002).

1.3. 11β-Hydroxysteroid Dehydrogenases

As discussed earlier, there are multiple aspects of control of glucocorticoid action. A central aspect of this project is the pre-receptor control of glucocorticoid access to the adrenocorticosteroid receptors. Research over the last decade has identified two 11β-hydroxysteroids which have specific roles to play in this modulation. This project is focussed upon intracellular control by 11β-HSD1.

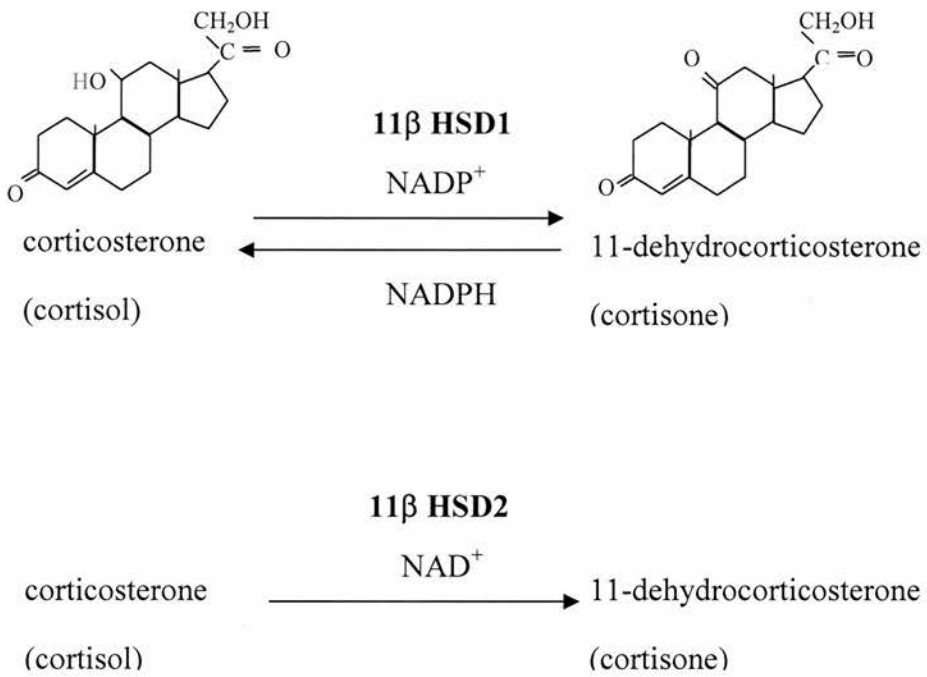

Figure 1-5: The 11β hydroxysteroid dehydrogenases

The 11β hydroxysteroid dehydrogenases (11β-HSD) are important pre-receptor controllers of glucocorticoid action. 11β-HSD1 is capable of the interconversion of glucocorticoids in rodents and humans (the human analogue is shown in brackets). 11β-HSD2 acts unidirectionally as an 11-dehydrogenase, reducing the levels of active glucocorticoid.

1.3.1. The Type 1 Isozyme

This enzyme was first identified in rat liver (Lakshmi 1985; Agarwal et al. 1989) and has been located as protein and mRNA in rodent adipocytes, rat testis and blood vessels (Jellinck et al. 1993; Stewart and Krozowski 1999; Brereton et al. 2001). Within the brain (mice, rats and humans), 11β-HSD1 has been demonstrated in the hippocamus, the cingulate gyrus and frontal cortex, as well as the PVN and cerebellar cortex (Moisan et al. 1990; Lakshmi et al. 1991). The role of this enzyme in this region has been hotly debated and would depend upon the predominant direction of dehydrogenase / oxo-reductase action.

The enzyme has been purified as a 34kDa membrane-associated microsomal glycoprotein (Lakshmi and Monder 1988), which is dependent upon NADP/H cofactor (Figure 1-5). Membrane association has appeared to be important for activity direction. The *in-vivo* direction of action has, until recently, been confused by experiments which showed predominant dehydrogenase activity in tissue homogenates and predominant reductase in intact cells, such as primary cultured rat hepatocytes (Jamieson et al. 1995) and hippocampal neurons (Rajan et al. 1996). This has been explained by the discovery that in human liver the enzyme exists as a membrane-bound, disulphide-linked dimer. The dimer exhibits co-operative 11β-oxoreducing kinetics with Michaelis-Menten 11β-dehydrogenation action (Maser et al. 2002). The affinity of the dimer for corticosterone (Km 42.8μM) is lower than for the 11-dehydrocorticosterone (Km 19.7 μM), but notable here is the difference in the Hill coefficient, 0.7 *vs* 2.7. Thus the enzyme is suited to amplifying active glucocorticoid from low levels of 11-dehydrocorticosterone. It appears that the

catalytic activities of this enzyme are focused in the lumen of the endoplasmic reticulum (C-terminus) (Odermatt et al. 1999) in contrast to 11β-HSD2.

1.3.2. The Type 2 Isozyme

Despite 18% sequence homology (Tsigelny and Baker 1995), this enzyme has a trans-membrane orientation reversed with that of the type-1 (Odermatt et al. 1999). Such a reversed orientation is consistent with its role as a pre-receptor "antagonist". This separate gene product is also active as a membrane-bound dimer and has a high affinity (nM Km) affinity for corticosterone. High levels are found in the kidney (Yau et al. 1991) and other mineralocorticoid target tissues (Roland et al. 1995; Robson et al. 1998). In addition, high levels are found in the placenta (Murphy et al. 1974; Michaud and Burton 1977).

Intrigue over the apparent aldosterone specificity of renal MR in the face of relatively high levels of corticosterone had sparked research into the 11β-hydroxysteroids. It happened that the isoform responsible for this action was identified after the liver-type 11β-HSD1 due to the earlier development of a type-1 specific probe (Edwards et al. 1988). In a history reminiscent of the GR / MR story it has taken some time to dissect their form and function (reviewed in (Seckl 1997).

As discussed, 11β-HSD2 shields local MR from circulating corticosterone (Edwards et al. 1988; Funder et al. 1988). Thus, the receptor is open to activation by aldosterone (not an 11β-HSD2 substrate) and this arrangement is found in salt regulation regions such as the distal, convoluted tubules of the kidney (Rundle et al.

1989) and the subcommisural organ, nucleus tractus solitaris (blood pressure) and the venrtromedial nucleus, amygdala (salt appetite) of the adult brain (Roland et al. 1995). Otherwise, 11β-HSD2 is largely absent from the adult brain.

It was also found that 11β-HSD2 may protect developing tissues from maternal corticosterone at the placenta (Murphy et al. 1974) and locally to give site specific protection (Brown et al. 1996). Glucocorticoids are generally associated with reduced mitosis and the promotion of differentiation. In the developing embryo, it is essential to control these processes. Some morphological changes of the cerebellum have been found in transgenic mice lacking the type-2 isozyme (Holmes et al, unpublished) and behavioural consequences of prenatal inhibition of 11β-HSD have been found in adult rats (Welberg et al. 2000). This developmental role for type-2 isozyme is likely to protect GR, in addition to MR.

It is of interest that a recent observation in an *in-vitro* cell model suggests that 11β-HSD2 interacts directly with the ligand-free MR complex in a manner which is inhibited by corticosterone(Odermatt et al. 2001). This suggests that the enzyme may only protect the MR from glucocorticoids at nadir levels.

1.3.3. Dynamic Regulation of Corticosterone by 11β-HSD1

It should be remembered that the pool of 11-dehydrocorticosterone might increase with the diurnal rise in corticosterone and after stress, because of the dehydrogenation of corticosterone by the 11β-HSD2 (Harris et al. 2001). Therefore, the *in-vivo* conversion of 11-dehydrocorticosterone to corticosterone by 11β-HSD1 is

potentially increased as feedback is required. In addition, protein and mRNA levels have been shown to be modulated by corticosterone, stress and other bio-signals (Low 1994a; Voice et al. 1996; Low et al. 1994b). Work in 2S FAZA hepatoma cells (Voice et al. 1996) showed induction of activity by dexamethasone and decrease by insulin which were transcription mediated. There was also a suggestion of a post-transcriptional inhibition by IGF-1.

Early *in-vivo* work (performed when it was still thought that 11β-HSD1 was a predominant dehydrogenase) found that chronic adrenalectomy decreased 11β-HSD1 mRNA in the hippocampus of rats (relative to sham operated controls) without affecting the generation of 11-dehydrocorticosterone in tissue homogenates. Activity and 11β-HSD1 mRNA levels were both increased by concommitant dexamethasone treatment (Low et al. 1994b).

A key question has to be the significance of this enzyme which appears to be regulated by substrate and other hormones. This was tackled by the generation of a transgene, null for 11β-HSD1, on a MF1/129 mouse background (Kotelevtsev et al. 1997). Study of mice homozygous for the null allele showed elevated nadir levels of plasma corticosterone with an early rise in evening levels (Harris et al. 2001). There was a decrease in GR mRNA in the PVN and an increase in nadir plasma ACTH. Stress and glucocorticoid feedback experiments in the same study suggested impaired feedback of the HPA axis with a delayed return to normal levels of plasma corticosterone and ACTH. It was postulated that the increased plasma corticosterone could be through increased sensitivity of the adrenal to ACTH.

In line with the peripheral distribution of 11β-HSD1, these mice demonstrated a significant metabolic phenotype. An apparent reduction in hepatic GR signalling (despite the elevated plasma corticosterone), lead to an impairment of gluconeogenic enzymes in response to fasting (Kotelevtsev et al. 1997) and a reduction in the glucose response to stress and a high fat diet. These results, together with an increased sensitisation of the liver to insulin (at re-feeding after fasting) and an 'improved' plasma lipid profile (*ad lib* fed) (Morton et al. 2001), point to a role for 11β-HSD1 in type II diabetes and cardiovascular disease.

1.3.4. Summary

Studies indicate that 11β-HSD1 acts as the predominant 11-dehydrocorticosterone reductase in the brain, locally amplifying the active glucocorticoid from inert 11-keto glucocorticoids. 11β-HSD1 has a significant effect upon HPA axis feedback. Its absence leads to increased plasma corticosterone in mice, yet paradoxically has been shown to protect aged mice in a hippocampal dependent learning task (the age-related impairment of which is glucocorticoid associated). 11β-HSD1 null mice also demonstrate a significant metabolic phenotype which could impact positively upon diseases associated with ageing.

Thesis Aims

It has been demonstrated in this researcher's laboratory that absence of the 11β-HSD1 conferred some protection from age-related deficit in a hippocampal-dependent task (Yau et al. 2001). A likely mechanism was reduced glucocorticoid levels in hippocampal cells which were sensitive to glucocorticoid –associated damage in ageing. Indeed, earlier work had demonstrated a role for 11β-HSD1 and amplified corticosterone in potentiating glutamate toxicity in primary cultured rat hippocampal neurons (Rajan et al. 1996). This thesis was designed to answer several questions arising from this work and provide early investigation into the largely unexplored role of glucocorticoid and 11β-HSD1 in the cerebellum.

1. Initially the thesis asked whether the protection seen in the aged 129/Ola, 11β-HSD1 knock-out mice (Yau et al. 2001) correlated with protection from age-related changes in molecular aspects of glucocorticoid signalling. This would be achieved by estimating levels of GR and MR mRNA in cells of hippocampi (from the behaviourally tested mice) by in-situ hybridisation. It was hypothesised that a decrease in GR and / or MR mRNA would be seen in the aged C57BL/6J control mouse (compared with the young) and that this change would be negated in the aged transgenic hippocampus. In addition, levels of 11β-HSD1 mRNA would be estimated in the young and aged control mice to test the hypothesis that an increase in 11β-HSD1 transcription with ageing resulted in a local increase in corticosterone, which then potentiated neurotoxicity. This experiment also sought to examine changes in GR and 11β-HSD1 mRNA in the cerebella of the same mice.

2. The study also sought to further explore the behavioural consequences of an absence of 11β-HSD1 in the aged mouse. Would the improved performance in a hippocampus-dependent task which had been seen in aged 129/Ola mice (Yau et al. 2001) also be seen on another suitable genetic background? C57Bl/6 mice (null for 11β-HSD1) were tested for spatial memory in a Y-maze. It was hypothesised, that an impairment in long-term memory would be seen in control, ageing mice (relative to young control mice) and that ageing 11β-HSD1 knock-out mice would be protected. In addition, it was hypothesised that a decrease in intra-cellular corticosterone would be associated with reduced anxiety. This was tested using an elevated-plus-maze and open field exploration. Finally, cerebellar related behaviour was explored using a Rota-rod. This was a novel study of glucocorticoid influence upon this paradigm and was more exploratory than designed to test a hypothesis.

3. The final experiment was designed to develop *in-vitro* models of enzyme activity in the frontal cortex and the cerebellum. A primary culture model had previously been developed for the rat hippocampus (Rajan et al. 1996) and used to examine 11β-HSD1 contribution to excitotoxicity. The question of enzyme direction (reductase or oxidase) also remained for the frontal cortex and cerebellum. Establishing the direction of activity in such *in*-vitro models would contribute to an understanding of the activity in the adult brain. The thesis sought to test the hypothesis that 11β-HSD1 was acting as an 11β-reductase in these other expressing regions.

Chapter 2

Molecular Aspects of 11β-HSD1 in the Ageing Hippocampus and Cerebellum

2.1. Introduction

In order to determine the role of 11β-HSD1 *in vivo,* 11β-HSD1 null mice (referred to hereafter as 11β-HSD1 knock-out) were created by replacing exons 3 and 4 of the 11β-HSD1 gene with a neomycin resistance cassette (Kotelevtsev et al. 1997). There was no overt phenotype (e.g. no changes in offspring viability), but these mice were shown to have increased adrenal size and increase basal (AM) plasma corticosterone levels. Further work suggested that there was reduced GR signalling in the pituitary and impaired HPA axis negative feedback (Harris et al. 2001). This complemented evidence that 11β-HSD1 was acting as a corticosterone reductase converting 11-dehydrocorticosterone to active corticosterone in the hippocampus (Rajan et al. 1996). The question was raised whether reducing the activity of 11β-HSD1 in the hippocampus could help to prevent age-related memory deficits, which were known to be corticosteroid related (discussed in Chapter 1). Indeed, 11β-HSD1 knock-out mice show attenuated glucocorticoid- and age-associated learning impairments in the watermaze (Yau et al. 2001). Pointing to a potential mechanism for this attenuation, inhibition of 11β-HSD1 in the adrenalectomised rat has been reported to reduce 11-dehydrocorticosterone-potentiated kainic acid neurotoxicity within the hippocampus (Ajilore and Sapolsky 1999). It can be concluded from these results that the local level of corticosterone in the neurons of the hippocampus (despite elevated plasma corticosterone) had somewhat protected them from the deleterious effects of high corticosterone in ageing.

This chapter was designed to determine whether the absence of 11β-HSD1 would protect against age-related changes in the mRNA of GR and MR in the hippocampus.

This would be achieved by in-situ-hybridisation in brain sections from young and aged mice, comparing those of wild-types with age-matched 11β-HSD1 knock-outs. Several studies have found reductions in corticosterone receptor mRNA in the ageing hippocampus (refer to table 1-1). There is evidence that hippocampal binding sites are also progressively reduced (Hassan et al. 1999; Murphy et al. 2002). Hippocampal MR and GR mRNA have been increased through monoamine targeted antidepressants (Seckl and Fink 1992). Antidepressants have also been used to normalise HPA axis function in aged, cognitively impaired rats (Yau et al. 1995; Rowe et al. 1998). It is known that GR expression is regulated by GR (Rosewicz et al. 1988) and MR signalling (Chao et al. 1998). It is possible that by altering local intra-cellular levels of corticosterone, 11β-HSD1 may alter expression of either receptor and mediate changes in local ageing. In addition, were there changes in 11β-HSD1 mRNA in the ageing brain, which could contribute to changes in glucocorticoid signalling? The opportunity could also be taken to explore possible age-related changes in GR mRNA in the cerebellum, a question which has never been addressed. mRNA was analysed by in-situ hybridisation histochemistry, which would give a quantitative estimate at the cellular level.

Chapter outline

This chapter will outline the general methods involved with in-situ-hybridisation of this type of tissue (section 2.2) and then will go on to describe the in-situ-hybridisation of the 129/Ola brain sections (section 2.3) for 11β-HSD1, GR and MR mRNAs in the hippocampus, and 11β-HSD1 and GR mRNA in the cerebellum.

Some repeatedly used techniques and materials are detailed in Appendix A and details for riboprobe generation (specific to the probe) are detailed in Appendix B.

2.2. General Methods and Materials

2.2.1. In-situ hybridisation histochemistry

This technique involved the regulated hybridisation of labelled fragments of RNA or DNA which were specifically complementary to sequences of mRNA of interest. This permitted localisation of mRNAs and the semi-quantitative estimate of mRNA levels at a cellular level. The probe fragments could be labelled by several different methods, appropriate to the tissue, required results and the laboratory facilities. The details for solution preparations can be found in Appendix A.

Generating 35S-labelled RNA probes

Creating the DNA template - The probes used in this experiment were for mouse MR, rat GR (appropriate for use in mice) and mouse 11β-HSD1. Anti-sense (AS) and control sense (S) probes were used in each experiment. Sense controls are usually hybridised with the labelled RNA complementary to the cDNA anti-sense strand. They should be roughly the same size and GC (bases) content, to replicate probe conformation and melting point.

The templates were held as laboratory resources. The DNA was subcloned into appropriate plasmid vectors (refer to Appendix B for template details) and amplified through bacterial replication (refer to Appendix A for procedural details). The DNA was extracted from the cells, purified through a CsCl – ethidium bromide gradient and then the template DNA digested from the plasmid by a specific DNA ligase

(refer to Appendix B), The integrity of the DNA at various stages of purification was checked on an agarose gel (Figure 2-1).

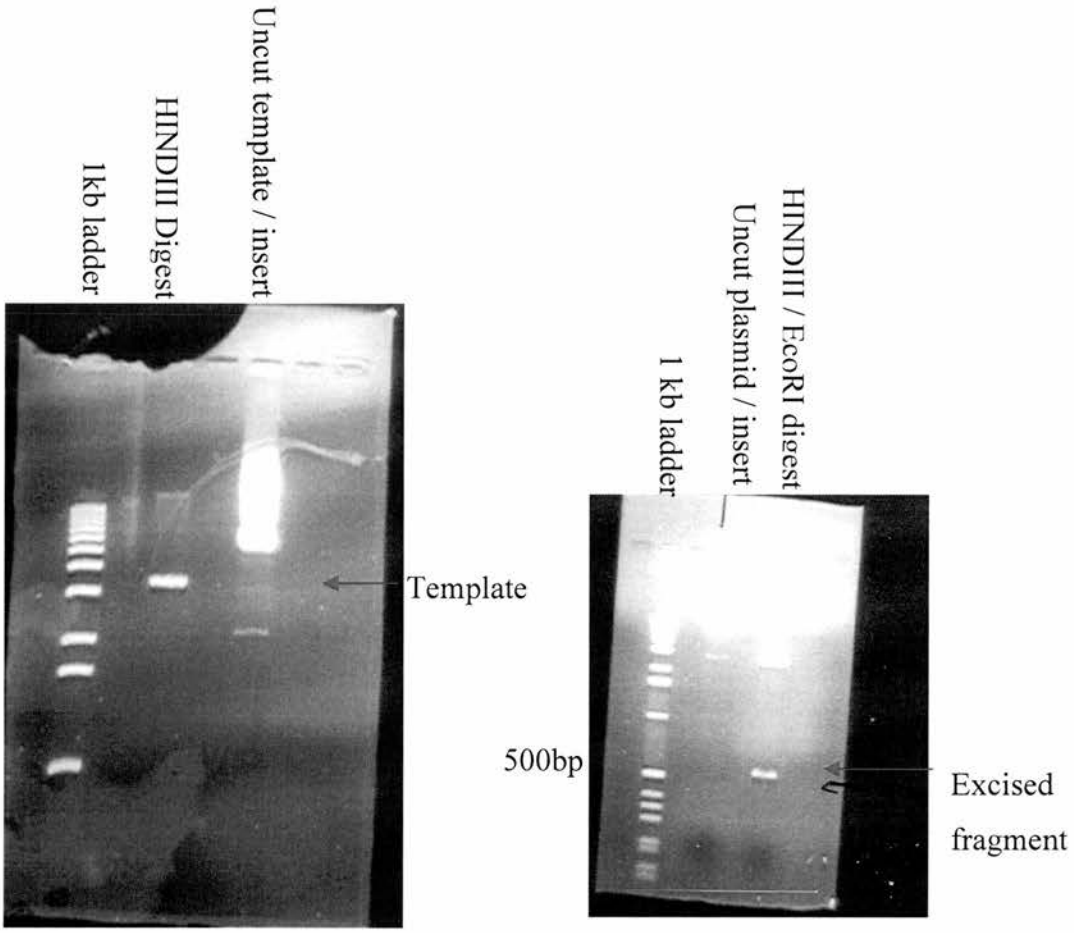

Figure 2-1: Examples of agarose gels checking template digestion and insert integrity

The images show agarose gels used to check template digestion (left) and insert integrity (right) for DNA sense template for the production of anti-sense MR riboprobes.

Creating the ^{35}S-labelled riboprobes - The DNA template was incubated with appropriate RNA polymerase in the presence of ^{35}S-labelled nucleotide mix (refer to Appendix A). The product was purified on a Nick column (Amersham) and the yield

estimated by scintillation (1μl elutant in 1ml scintillation fluid should give 0.5 x 10^6 cpm on a beta counter).

The probe integrity was checked on a urea/ acrylamide gel (refer to Appendix A). The RNA probe is sensitive to RNase contamination and was kept on ice when thawed, stored at –20°C and freeze-thawing was avoided. Probe were used within 10 days of preparation.

Hybridisation in fresh-frozen sections

Solutions - The general solutions are detailed in Appendix A. All solutions and apparatus for use up to the washing stage were kept as RNase-free as possible. RNases are common in the general laboratory environment and can be resilient. Glassware was washed and baked at 200°C for 2 hours. Glass and plastic-ware were treated by soaking in 10M NaOH, treatment with an inhibiting preparation such as RNase Zap (Ambion) or soaking in diethyl pyrocarbonate (DEPC) (Sigma). Water for the preparation of solutions was purchased RNase-free or double-distilled water was treated with DEPC.

Tissue preparation - To prevent the degradation of mRNA, samples were removed and frozen as quickly as is possible, in a manner suitable for the tissue. Rodent brains were well preserved by placing then directly onto powdered dry-ice in an insulated container. Freeze-thawing was avoided (but samples last well stored at –80°C). Tissue was cut on a cryostat at 7-10μm at approximately -18°C, onto prepared slides and stored at -80°C.

The sections of tissue were fixed and prepared for the in-situ to minimise background caused by non-specific binding and to maximise penetration and retention of the probe (refer to appendix A). The details of the preparation procedure may have to be adapted to the tissue and the length and nature of the probe.

Prehybridisation and hybridisation

The tissue may be incubated in a pre-hybridisation stage to reduce non-specific binding. The pre-hybridisation and hybridisation steps were performed in an RNase-free humidifying chamber (refer to Appendix A). Humidity was maintained by lining the base of the chamber with a double layer of Whatmann paper soaked in Box Buffer. Pre-hybridisation buffer (50% 2 x pre-hybridisation buffer, 50% deionised formamide) was applied to the sections on the slide (200µl per slide) such that the sections was covered but buffer did not touch the slide edge. The slides were incubated in the sealed chamber at 50°C (2-3 hours).

The probe was denatured in the hybridisation buffer before applying to the section. For targets such as rat GR or MR, probe was added at $10x10^6$ counts per ml. The buffer contained 50% deionised formamide, an appropriate volume of probe RNA and the volume completed with the 2 x hybridisation buffer. The hybridisation buffer (before the probe is added) was warmed to 70°C and the probe added (incubated for 10 minutes). It was then put on ice for 2 minutes and 10µl/ml 1M DTT added to prevent renaturation. The pre-hybridisation buffer was removed from the slides, leaving a dry border. The sections were not allowed to dry and 200µl per slide of the appropriate hybridisation buffer was added. The slides were replaced and incubated

at 50°C in the sealed chamber (the precise temperature depends upon the probe used) for 12 to 16 hours.

Washing un-hybridised probe - After hybridisation the excess probe was removed by digestion and washed. It was now not necessary to protect the sections from RNases, but RNase treatment dedicated apparatus and work areas were used to prevent contamination of other experiments. The slides were washed three times in 2xSSC (in distilled water) and 200µl RNase A (20µg/ml in RNase buffer) was added to each slide to remove non-hybridised RNA. The slides were incubated at 37°C (1hour) in a humidifying chamber with a Whatmann liner soaked in RNase buffer. They were then put through a series of washes of increasing stringency (refer to Appendix A) and dehydrated through an ethanol in ammonium acetate series.

Visualisation

Rapid but un-detailed results were achieved through use of the phosphorimager. This was useful for confirming whether adequate hybridisation had been achieved. Results for analysis could also be obtained through autoradiography. The slides were placed against film for a period of 2 to 3 weeks and the film developed. Sections of brain could be analysed, over a light box, by eye or through the use of an image analysis system. During the course of this project, such a method was used to back-up analysis at the cellular level, but was inferior in its detail and did not have to be called upon.

The use of liquid emulsion - The preferred method was computer assisted analysis at the microscopic / cellular level. To achieve this, the slides were incubated in the

dark in photo-sensitive NTB2 emulsion (Amersham) and the slides developed after an appropriate period of time. In the dark room, emulsion was melted at 42°C, an equal volume of distilled water was incubated simultaneously. The melted emulsion was 50% diluted with the water and gently mixed (bubbles were avoided). The tube was left 10 minutes to settle and gently poured to fill a slide mailer. Bubbles were removed.

The experimental slides were dipped (this was controlled for consistent results), the excess emulsion were drained quickly and the slides racked horizontally. The slides were left until dry and placed into a photo-sealed black box, protected from light and stored at 4°C. As with autoradiography, determination of the length of incubation time was based upon experience. Ideally, a linear relationship between β-particle emission and silver grain density would be achieved. It was useful to include up to 3 extra positive control slides in any experiment to test incubation time and the quality of development solutions.

Development was also performed in the dark-room on slides which have been brought to room temperature. Troughs were prepared: 50% D19 (Kodak) developer; water; 25% fixer; water. Solutions were brought to and maintained at 15°C (essential) and the slides taken through the series (4 minutes, 10 seconds, 5 minutes, 5 minutes respectively). It was important that solutions should not be used for more than 2 racks of slides. Slides could be air dried in the light.

Counterstaining - The slides were counterstained for cellular visualisation and to provide a background for the silver grains. There is a vast list of potential

counterstains and deciding which to use will depend upon laboratory culture, the cells which need to visualised and aspects of the specimen fixation stage. For the purpose of this experiment Pyronin was used.

Analysing silver grain density - Grain density was analysed using the dedicated computer-based image analysis programme KS-300 (Imaging Associates). Analysis was performed upon a colour video image at x500 magnification. It was important at the beginning of a count for each experiment to establish counting parameters. The object was to maximise the number of grains identified by the system whilst minimising the influence of dark areas of counterstain. Grains were identified by a criterion level of rate of change of black:white on a monochrome, extracted image, therefore dark areas of counterstain could also be counted. Developing this aspect would include image modulation within the system and adapting the thresholds of counting, however, information was maximised by manipulating the image at the microscope, before it reached the computer.

As grains were formed within the 3-dimensional thin layer of emulsion and image analysis was performed in 2-dimensions, a series of algorithms were applied to clumped areas of grains to predict the number within a group.

Getting representative counts - Any count should control for background levels of grains. Sites for background should be predetermined and consistent; some would argue that it should be over non-expressing cells. In addition, as β-particles are emitted in 3-dimensions and thus the grains do not always appear above hybridisation site, any counting should include a perimeter which would include such escaped grains. A caveat of this method, is the contamination of grain numbers by

those of neighbouring cells. For this reason, areas of anatomical regions to be counted should be made as consistent as possible (in terms of cell density). This reduces sampling error between subjects, but does mean that comparison between dense regions such as the hippocampal dentate gyrus and sparse regions such as the CA3 is impaired.

The number of cells counted to obtain statistical reliability will depend on the variability of expression, however a minimum of 10 cells is recommended. The researcher may choose to count 'expressing cells' or to count all cells in a region and include numbers of expressing cells within the results.

2.3. In-situ Hybridisation for GR, MR and 11β-HSD1 mRNAs in the Ageing 129/Ola Mouse Brain. The Influence of 11β-HSD1 upon Age-related Changes.

2.3.1. Methods

Animals

Behaviourally tested male mice (Yau et al. 2001) were 11β-HSD1 knock-out and wild-type on a 129/Ola background. The 'young' were 4-7 months and the 'aged' were 18-20 months for each genotype, with 4-11 per group. The mice were culled 2 weeks after watermaze testing, between 08.00 and 10.00 hours in random order. The brains were excised and rapidly frozen, then stored at –80°C (performed by Dr J. Yau and Mrs J. Noble). Sections were cut as described. The hippocampal region was cut at 10μm and the cerebellum at 7μm, to compensate for the dense granular cell layer at visualisation.

Hybridisation

GR, 11β-HSD1 and MR (sense and antisense) probes were generated using the protocol previously described in this chapter. The hybridisation was carried out as described (MR was not applied to cerebellar sections) and the slides for the GR and MR probes left for 2 weeks and those for the 11β-HSD1 left for 3 weeks. The slides were then processed as previously described.

Analysis

For each probe, 10 cells were counted from each hippocampal half of two sections. Results were analysed based upon the mean count for each slide and groups were

compared by ANOVA (age and genotype as the independent variables) with LSD post-hoc analysis as appropriate. Results are presented as group means ± s.e.m.

2.3.2. Results

The sense controls generated no signal in the areas of interest (Figure 2-2) and signal from the antisense was judged to show specific hybridisation of the probe.

11β-HSD1 mRNA in the hippocampus

Expression of 11β-HSD1 was observed in the hippocampus (Figure 2-2) at relatively high levels in the cells of the CA3 region. However, expression in the CA1 and DG was relatively low. This was with the exception of a number of high expressing cells around the pyramidal layer and within the granular molecular layer. High expressing cells could also be seen in the stratum radians, oriens, alveus and the polymorphic layer. These high expressing cells appeared to follow a pattern which was similar in each of the sections examined.

Expression of 11β-HSD1 mRNA in cells of the CA1, CA3 and granular molecular layer (G Mol) was analysed by counting grain expression over 10 expressing cells within each region. There was no change in the level of silver grains with ageing in CA1 ($F_{(1,14)}$ =0.53; p= 0.48), CA3 ($F_{(1,15)}$ =0.29; p= 0.6) or in G Mol ($F_{(1,15)}$ = 0.058; p= 0.81). There was no signal in the 11β-HSD1 knock-out sections, demonstrating specificity of the probe.

Figure 2-2: Expression of 11β-HSD1 mRNA in the hippocampus of young and ageing mice

(A) Dark-field micrographs (x50) of typical hippocampal sections from young and ageing mice. The sections display dense bright grains (^{35}S-labelled anti-sense riboprobes to 11β-HSD1 mRNA) in the Cornu ammonis 3(CA3) and granular molecular (G Mol) region of the dentate gyrus. (B) When grains per neuron were counted on bright-field video image (using a dedicated image analysis software (KS300)), there were no differences in expression of 11β-HSD1 mRNA in CA1, CA3 or the G Mol. (C) Dark-field micrographs of negative controls showing low non-specific binding with sense riboprobe and an absence of labelling in the knockout.

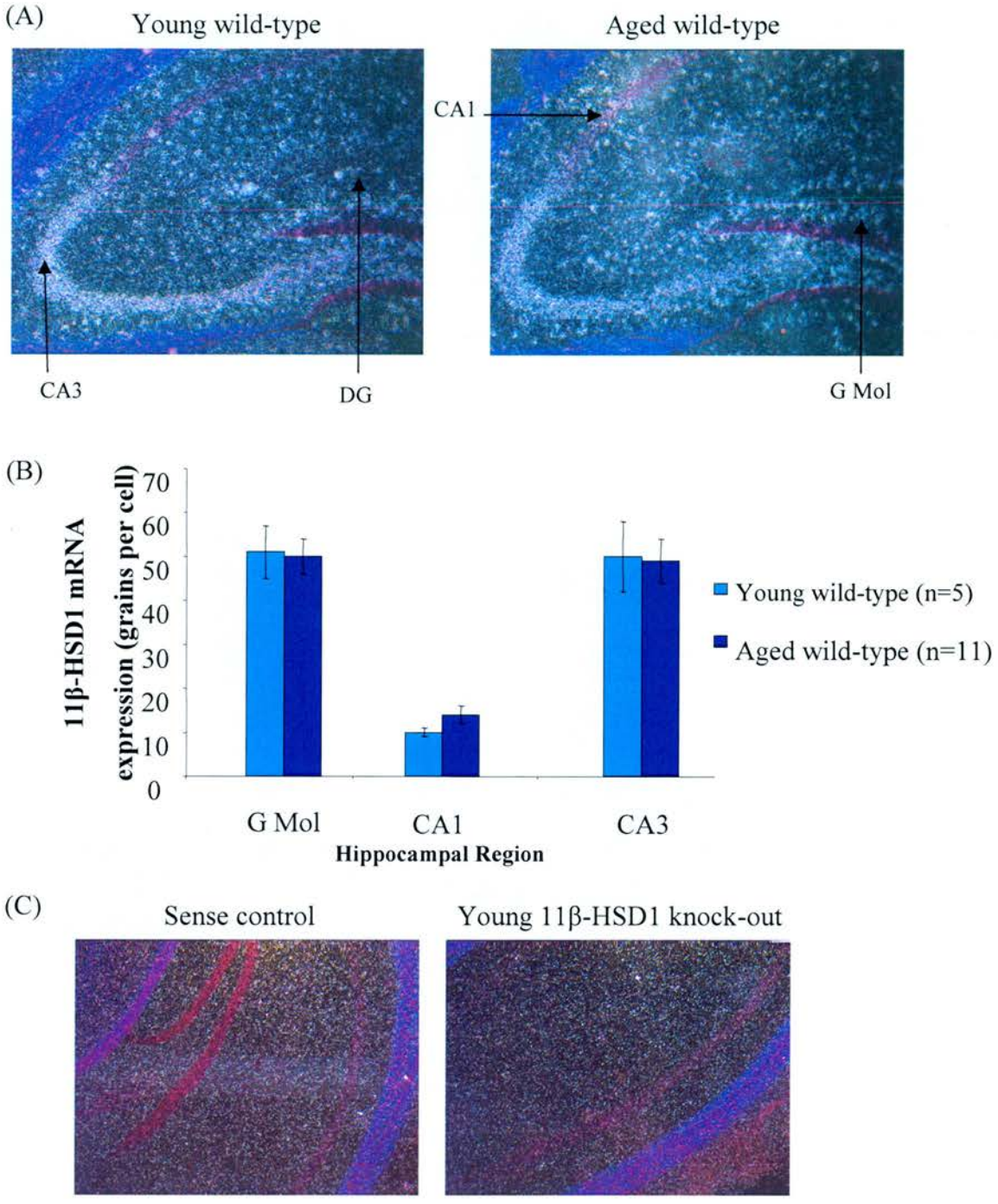

Figure 2-2: Expression of 11β-HSD1 mRNA in the hippocampus of young and ageing mice

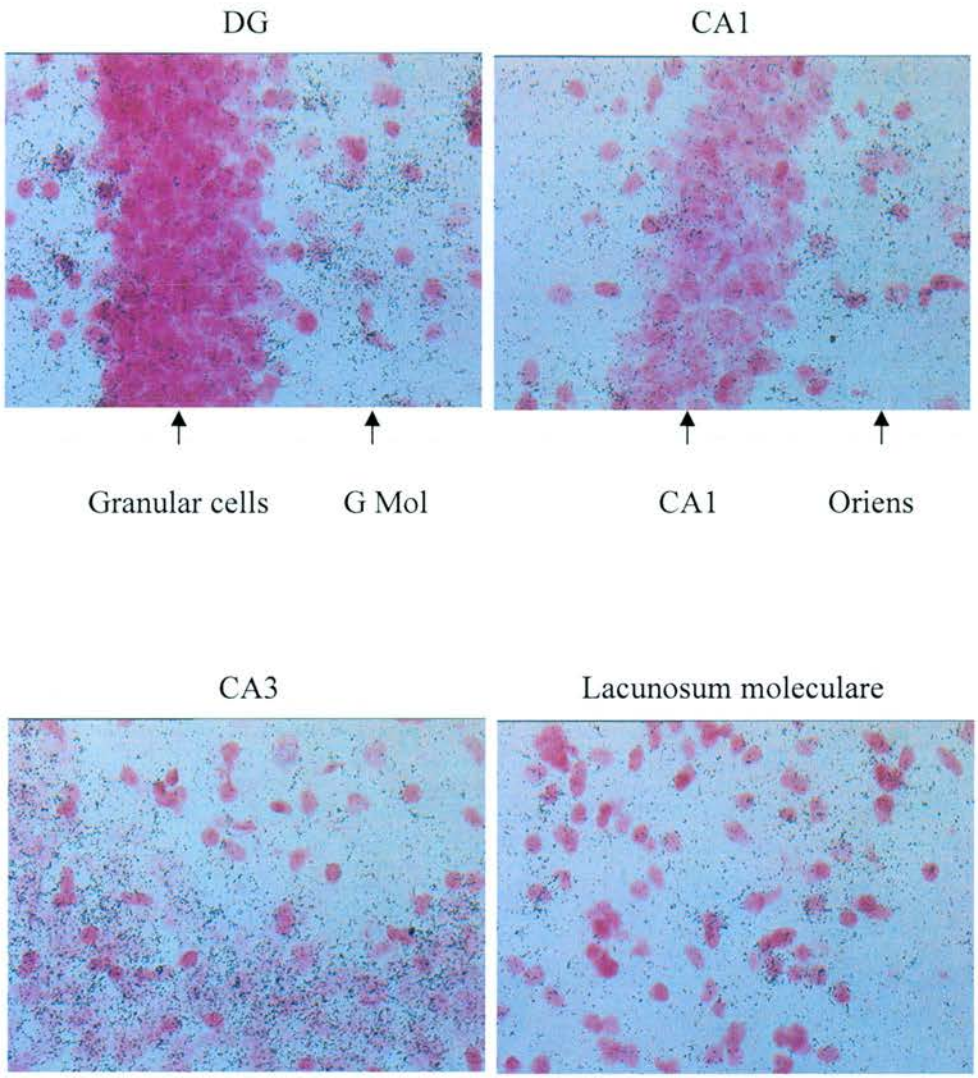

Figure 2-3: Bright-field micrograph of in-situ-hybridisation for 11β-HSD1 in regions of a typical hippocampal section from a young wild-type mouse

The black grains show ^{35}S-labelled complementary RNA (magnification x400). Expression in most cells of the DG, CA1 and Lacunosum moleculare are low in expression with some high expressing cells. The cells of the CA3 were high expressing. Grains were counted within a predefined diameter of circle which was centred upon an expressing cell nucleus (shown in pink) and the count was controlled against a background value. A mean average of 10 cells was taken for each region of each section.

Glucocorticoid receptor mRNA in the hippocampus

Expression of GR mRNA could be seen in cells of the CA1, CA3, DG and, to a lesser extent, throughout the polymorphic and molecular layers (Figure 2-4A, Figure 2-5). Expression in cells of the pyramidal and granular layers was analysed from 10 cells per section and results are expressed as previously.

GR mRNA expression was analysed in cells of the CA1, CA3 and DG. Analysis by ANOVA showed a decrease in GR mRNA in the CA1 with ageing (Figure 2-4B) and a decrease in the absence of 11β-HSD1 ($F_{(1,21)}$=12.2; $p<0.01$) and with increase in age ($F_{(1,21)}$=7.86; $p<0.05$). There was a significant interaction between these factors ($F_{(1,21)}$=4.27; $p<0.05$).

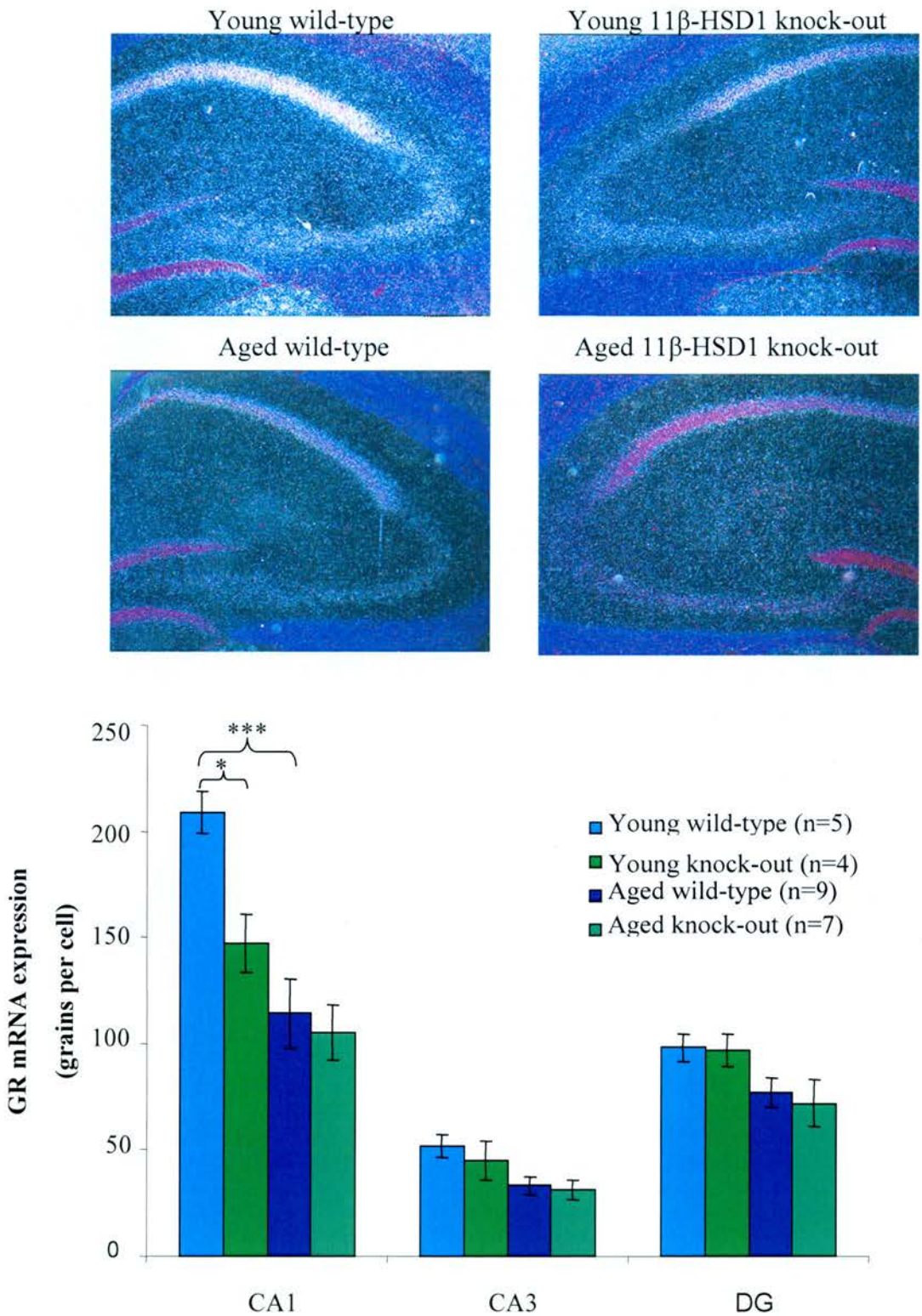

Figure 2-4: Effect of age on the expression of GR mRNA in the hippocampus from 11β-HSD1 knock-out mice and wild-type controls

Dark-field micrographs (magnification x50) showing silver grains from an in-situ-hybridisation for GR mRNA. The chart shows silver grain levels for GR mRNA in cells of the hippocampus. LSD post-hoc analysis: *= $p<0.05$, *** = $p<0.0005$

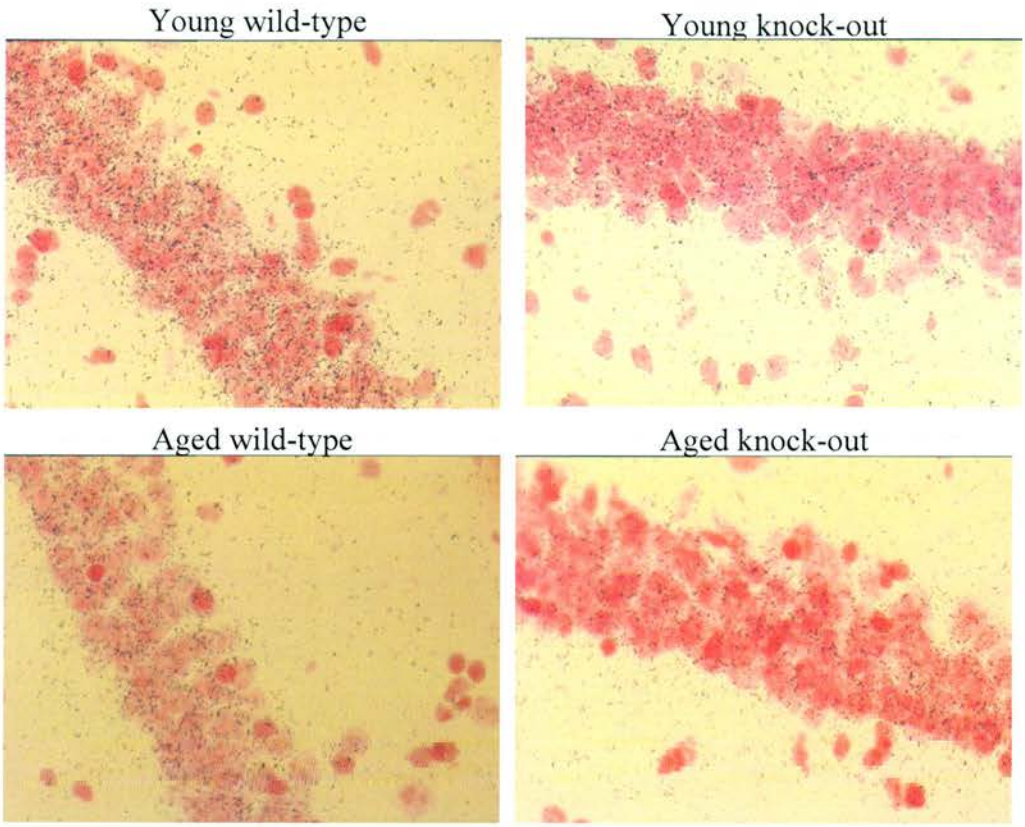

Figure 2-5: Bright-field micrographs of in-situ-hybridisation for GR mRNA in CA1 region of the hippocampus from representative sections of young and aged 11β-HSD1 knock-out and wild-type mice

(Magnification x400). A reduction in GR mRNA can be seen from the young wild-type with loss of 11β-HSD1 and with ageing. Most cells of the CA1 are expressing GR mRNA.

Mineralocorticoid receptor mRNA in the hippocampus

Expression of MR mRNA could be seen in cells of the pyramidal layer and the dentate gyrus and was counted in the same manner as for the GR (Figure 2-6). Unlike the GR, there was no expression in cells of the molecular layers.

The same regions were used to analyse levels of MR mRNA as for the GR mRNA. An increase was found in the DG region with absence of 11β-HSD1 ($F_{(1/22)}$=4.66; $p< 0.05$), but there was no effect of ageing ($F_{(1/22)}$= 0.13; p= 0.72).

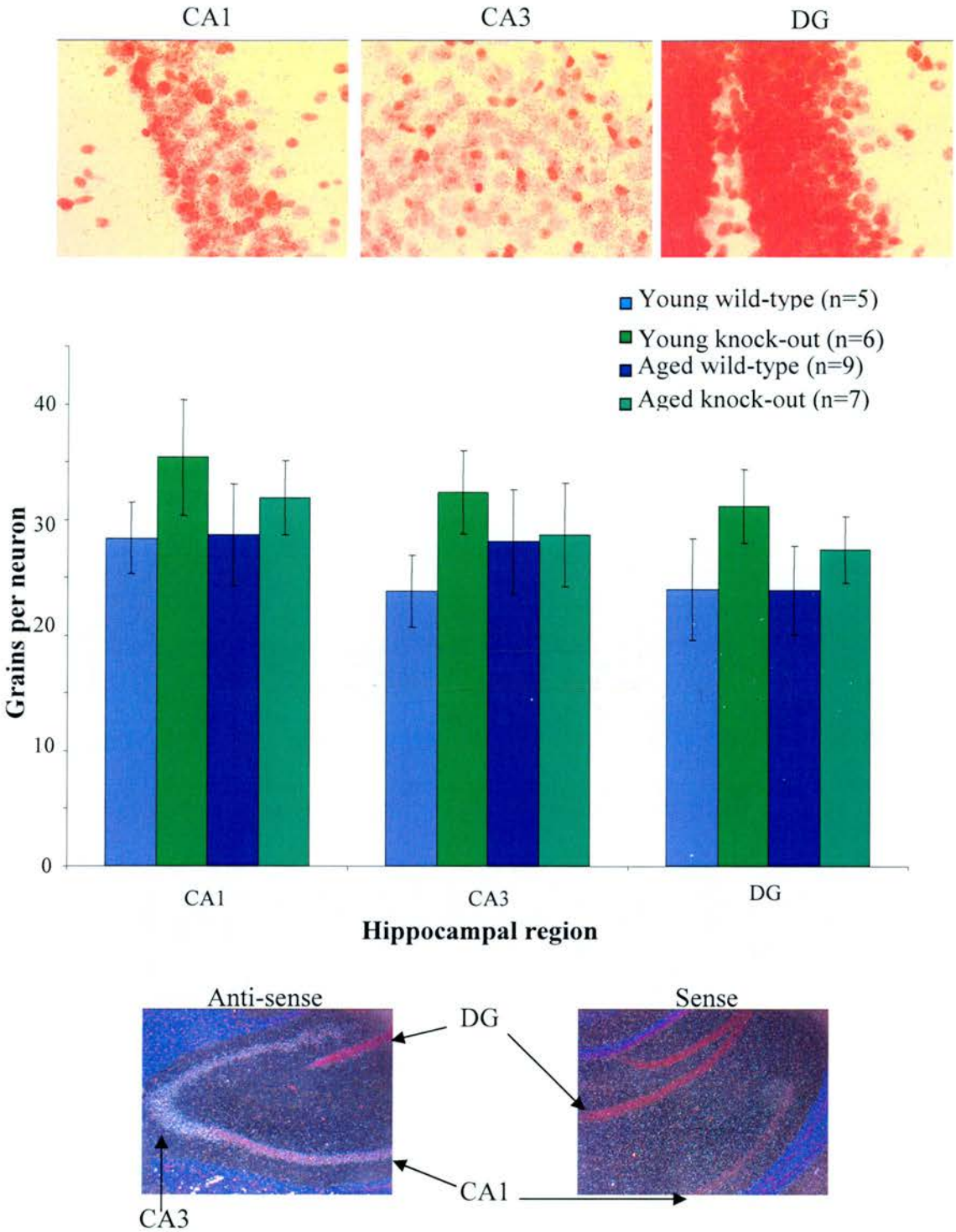

Figure 2-6: Expression of MR mRNA in the mouse hippocampus of young and aged 11β-HSD1 knock-out and wild-type mice

Bright-field micrographs (magnification x400) of analysed hippocampal regions from typical young wild-type 129/Ola. The chart shows the number of grains per cell. Dark-field micrograph (magnification x50) of anti-sense and sense control for ^{35}S riboprobe, demonstrating specific binding of the antisense probes.

11β-HSD1 mRNA in the cerebellum

11β-HSD1 mRNA expression could be seen in all lobules of the cerebellum visualised. In finer detail, expression was seen in Purkinje neurons and cells of the granular layer (Figure 2-7) and in discrete cells of the molecular layer, which remain unidentified. It was notable that expression was heterogenous throughout the granular layer, with low, punctuated expression in most of the inner layer and high expression proximal to the Purkinje layer. Expression in a minimum of 10 Purkinje neurons and 10 cells from the proximal Granular layer was counted in lobules 10, 4 and crusiform (Figure 2-8).

There was no change in the 11β-HSD1 mRNA in the Purkinje neurons or in the granular layer with ageing. There did appear to be a trend for a decrease in all regions examined, but this did not reach significance.

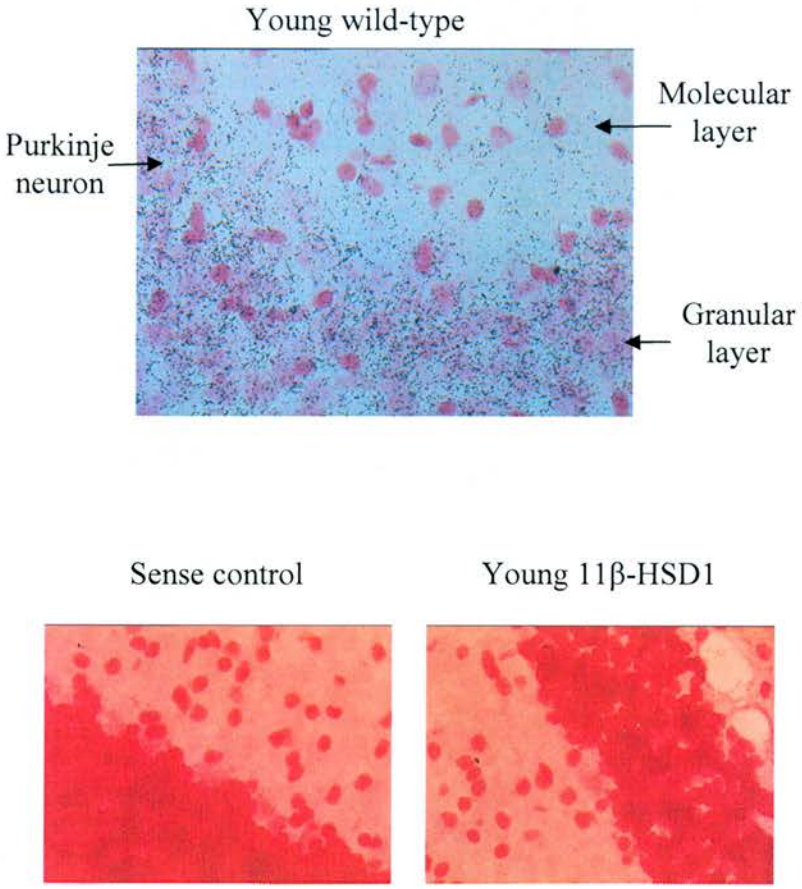

Figure 2-7: Expression of 11β-HSD1 mRNA in the mouse cerebellum of young and aged wild-type mice

The upper image shows grains (labelled complementary RNA) (magnification x400) in a typical young mouse cerebellum. The lower images show a lack of signal in a sense young wild-type control and a young 11β-HSD1 knock-out.

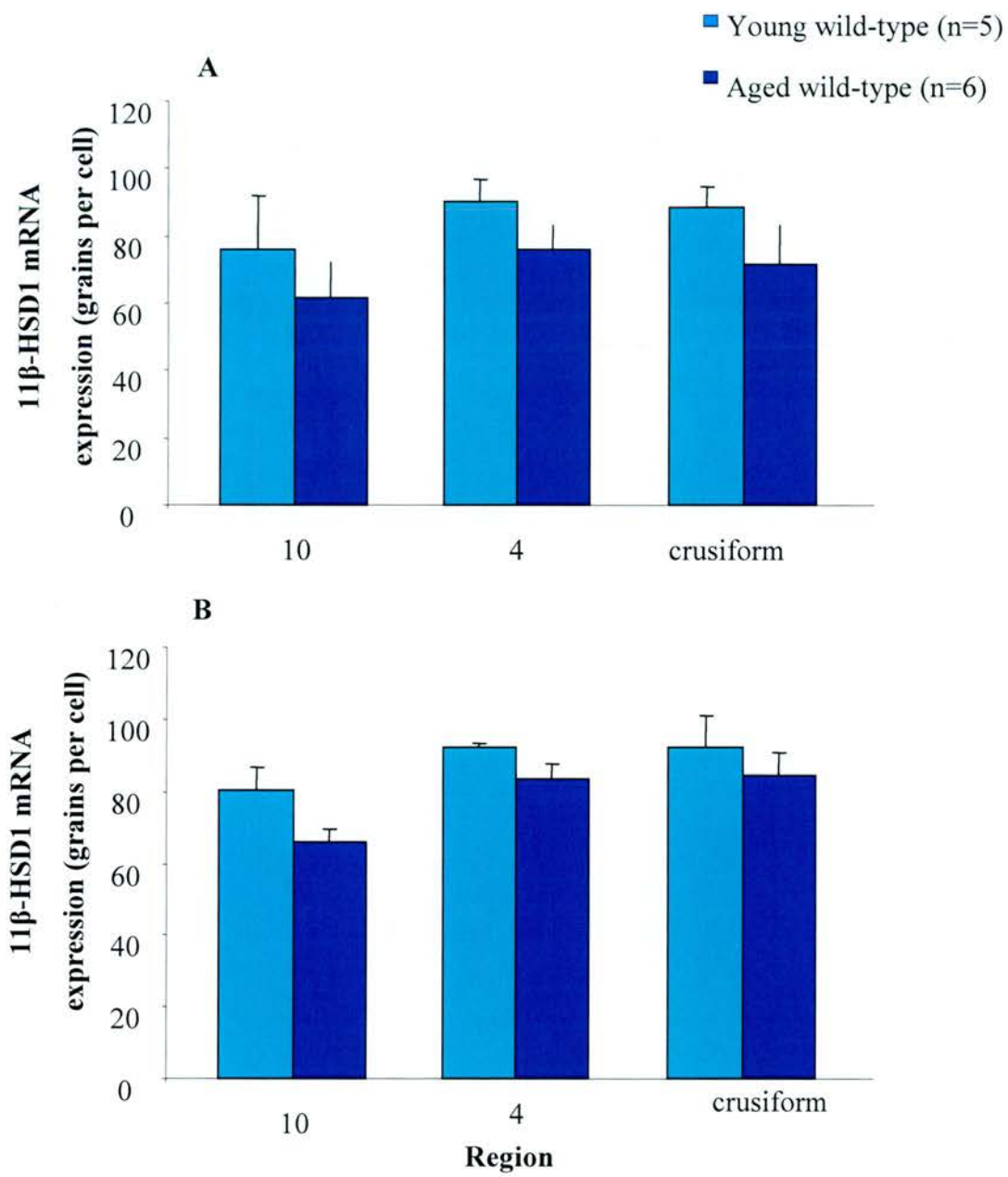

Figure 2-8: Levels of 11β-HSD1 mRNA in the cerebella of young and aged 11β-HSD1 knock-out and wild-type 129/Ola mice

Chart A shows expression in Purkinje neurons. Chart B shows expression in the outer granular layer. There were no differences with ageing.

Glucocorticoid receptor mRNA in the cerebellum

GR mRNA expression was found in all lobules visualised and was wider than that for 11β-HSD1 (Figure 2-9). Relatively even expression could be seen throughout the Purkinje neurons, but in the young wild-type mice it was noted that expression was higher in the inner than the outer granular layer. These layers were counted separately. Expression could also be seen in cells of the molecular layer, but these were not counted.

ANOVA analysis revealed no effect of age upon GR mRNA in any of the regions examined (Figure 2-10). In addition, there was no change in GR mRNA in the Purkinje cells with loss of 11β-HSD1. However, there was a significant decrease in GR mRNA in 11β-HSD1 knock-out mice throughout the granular layers of the lobules examined. There were significant changes in the outer granular layer of lobule 10 ($F_{(1,20)}=4.68$; $p<0.05$) and of the inner granular layer ($F_{(1,20)}=10.66$; $p<0.005$). This was also true for lobule 4 outer layers ($F_{(1,19)}=8.51$; $p<0.01$) and inner ($F_{(1,19)}=23.29$; $p<0.001$) and for the comparable crusiform layers (($F_{(1,19)}=9.04$; $p<0.01$) and ($F_{(1,19)}=6.58$; $p<0.05$)). LSD –post hoc analysis revealed significant differences between the different genotypes regardless of age.

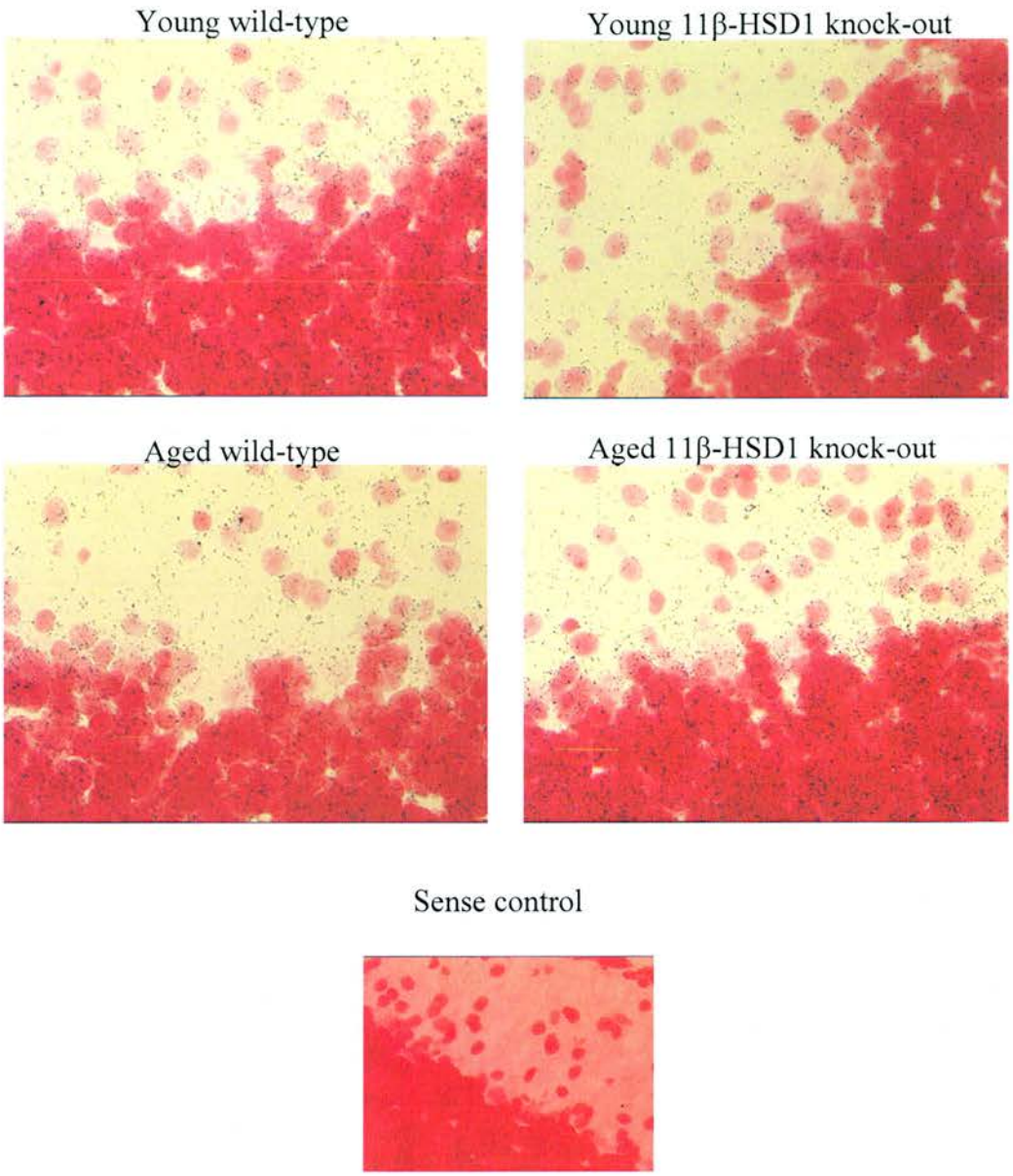

Figure 2-9: Expression of GR mRNA in the cerebellum

Bright-field micrographs (magnification x400) of in-situ-hybridisation for GR mRNA in typical, lobule 10 cerebellar sections from different experimental groups and a sense control. The black dots are silver grains.

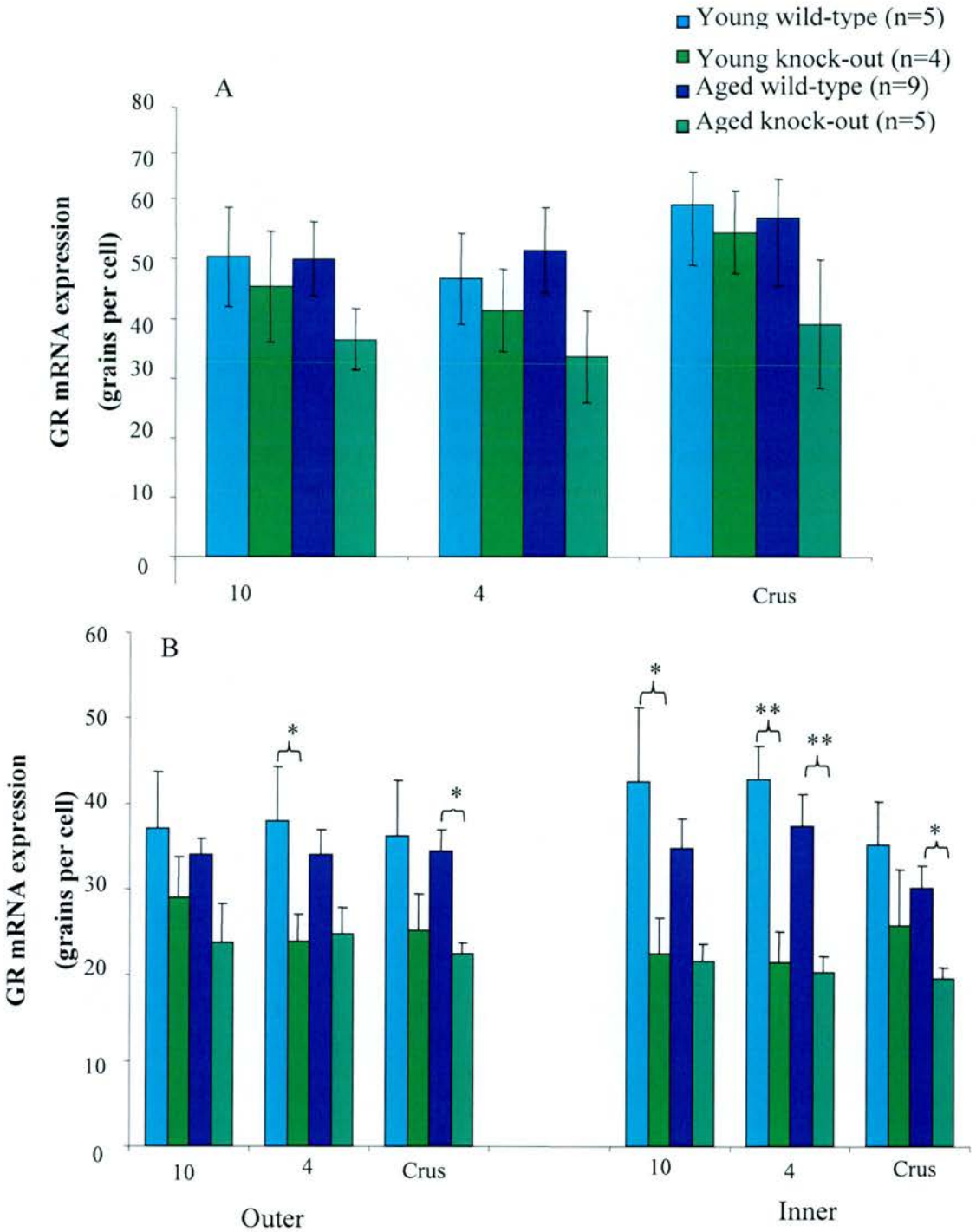

Figure 2-10: Levels of GR mRNA expression in the mouse cerebellum of young and aged 11β-HSD1 knock-out and wild-type 129/Ola mice

Chart A shows grain counts over the Purkinje neurons in lobules 10, 4 and the cruciform of mouse cerebella from the experimental groups. Chart B shows grain counts from the inner and outer granular layers. *=$p<0.05$, **=$p<0.005$ LSD post-hoc analysis.

2.3.3. Discussion

The results of the above in-situ-hybridisation experiments generated quantitative and qualitative information. It was of interest that there was no significant decrease in 11β-HSD1 mRNA with ageing, in any region examined. This question was central to the project. A change may have suggested a mechanism for age-related changes in GR. This, however, does not preclude a change in enzyme activity.

11β-HSD1 mRNA in the hippocampus

There was no effect of age upon the levels of 11β-HSD1 mRNA found in any region of the hippocampus. Although 11β-HSD1 mRNA has been shown to be increased by glucocorticoid via GR (Low et al. 1994b), there does not appear to have been such a response in the face of elevated corticosterone in the ageing group. It is possible that the change in plasma corticosterone is not enough to achieve this effect or that such an effect is species dependent. The results suggest that local changes in 11β-HSD1 mRNA are not responsible for changes in glucocorticoid signaling in the ageing hippocampus. The reduced corticosterone found in hippocampi of aged, 11β-HSD1 knock-out mice (Yau et al. 2001) suggest that the enzyme is still active and effective in the older animal, it remains to be seen whether activity is equivalent in young and older brains. It is notable that in studies looking at 11β-HSD1 in the hippocampus (in the short-term) mRNA levels have not always paralleled enzyme activity (Jamieson et al. 1999), suggesting changes in translation or protein stability.

It was of interest that 11β-HSD1 mRNA was found in a consistent pattern in cells of lacunosum moleculare and the oriens. These regions contain interneurons which

modulate the activity of the perforant pathway and the Schaeffer collaterals respectively (Johnston and Amaral 1998) and are rich in astrocytes.

Glucocorticoid receptor mRNA in the hippocampus

The in-situ-hybridisation for GR mRNA in the hippocampus showed a pattern for reduced GR mRNA with absence of 11β-HSD1 and with age, however this only reached significance in the CA1 region. It is of interest that there was a decrease in GR (Figure 2-10) in the CA1 region which expressed the least 11β-HSD1 mRNA of any of those tested. This could suggest a GR-mediated down-regulation of the GR mRNA in response to the high circulating corticosterone (Yau et al. 2001) rather than directly due to the absence of local 11β-reduction. Such down regulation has been previously shown (Paskitti et al. 2000). Correlations between GR mRNA and plasma corticosterone levels (obtained at the time of sacrifice) would be useful, in order to examine changes in hippocampal feedback upon the HPA, but are outside the remit of this thesis. The reduced GR mRNA in the young knock-out does suggest that the hippocampal inhibition of the HPA axis could be impaired and contributing to the increased drive of corticosterone production (Yau et al. 2001). In the current study, there was no difference in the GR mRNA seen between aged groups. These groups also showed equivalent nadir plasma corticosterone (Yau et al. 2001).

It is possible that GR mRNA in the CA1 region is modulated by changes in neuronal input from regions with higher 11β-HSD1 or sensitivity to glucocorticoid changes, such as the subiculum or the cingulate gyrus. The principle for changes in hippocampal GR with modulated input was established in studies looking at lesioned inputs (Yau et al. 1994a). In addition, another glance at the light micrographs of

11β-HSD1 mRNA (Figure 2-3), shows unidentified cells of high 11β-HSD1 mRNA expression in the alveus, oriens, radiatum and the molecular layers. These cells may be influencing the pyramidal layer, directly or through modulation of the above mentioned inputs.

A recent report has given valuable insight into ageing effects upon GR. This study of the ageing rat hippocampus found decreased GR mRNA in the CA1 (not the CA3 or DG) and no change in the MR mRNA (Murphy et al. 2002). In a detailed analysis of the protein, they also found that there was no change in protein levels, but they did find impaired translocation of GR into the nucleus. Results indicated that this may involve changes in the stabilising heat-shock proteins 70 and 90. Thus Sapolsky's early findings that GR binding of ligand was not age-impaired (Sapolsky et al. 1983) may stand true and the deficit may be intermediate between ligand and DNA.

The mechanism of the decrease in GR mRNA (Yau et al. 2002) is not yet clear, but is understood to play a role in impaired hippocampal function in cognition and in HPA feedback. It is of interest that there was no significant age-related loss in the 11β-HSD1 knock-out groups. It may be that levels had reached a floor effect or an effect of reduced GR signaling in earlier life. Glucocorticoid-mediated age effects are thought to be GR rather than MR operated, Thus, a decrease in GR transcript in these cells may indeed protect them. The lack of a difference between the genotypes in the aged animals does suggest a floor effect (an age-related mechanism has been previously suggested), although work in humans suggests that it is some aspect of the change, rather than absolute values, in glucocorticoid signaling that is correlated with cognitive deficit (Lupien et al. 1999).

Mineralocorticoid receptor mRNA in the hippocampus

In contrast to the GR there was no significant change in MR mRNA within individual groups (Figure 2-6). An age-related decrease in hippocampal MR mRNA has been found in some studies (Yau et al. 2002), though this has not been a robust finding (Murphy et al. 2002). An increase in MR in the hippocampus of 11β-HSD1 knock-out mice (not found in this thesis), would have had significant implications for the explanation of the protected ageing in these mice. MR has been shown to have a neuroprotective role in the hippocampus (Gass et al. 2000; reviewed in Gass et al. 2001). An increase in MR mRNA could have suggested a neuroprotective mechanism.

MR protein has been shown to negatively autoregulate in the rodent (Kalman and Spencer 2002) and there appears to be more of an influence of post-transcriptional regulation in MR than GR . Thus, although there has been no change in mRNA, one should not assume that there has been no change in glucocorticoid action in the cell. As discussed in the introduction, it is understood that MR does not play a plastic modulatory role in the HPA axis because of assumed, almost full occupancy at basal corticosterone levels. However, several lines of evidence have argued against this. In an anxiety test in rats, results suggested a rapid, non-genomic MR–dependent mechanism (Smythe et al. 1997), this would not occur if the MR was fully occupied before stress. In addition, results from the study discussed above showed a decrease in MR protein after stress, thus MR is modulated by stress (Kalman and Spencer 2002). In the latter paper, the binding kinetics of MR and GR were theoretically re-

analysed and the conclusion drawn that MR may only be half occupied at basal levels.

Possible interactions of 11β-HSD1, GR and MR

In a previous study conducted in the author's laboratory, 11β-HSD1 mRNA was co-localised with MR and GR mRNA in the rat hippocampus (Low 1994b). The question has to be raised as to whether individual cells are indeed exposed to lower or to higher levels of active glucocorticoid in the 11β-HSD1 knock-out hippocampus. Cells which would normally express MR and 11β-HSD1 would be exposed to lower corticosterone in the 11β-HSD1 knock-out. If the cell co-expressed GR, this may result in an increase in the MR : GR signalling ratio. In light of the differing effects of GR or MR homo-dimers and of GR/MR hetero-dimers (e.g. effects upon 5HT-1A expression (Ou et al. 2001a)) this may be an important mechanism of change with loss of 11β-reduction.

Thinking about potential effects in the regions of the hippocampus, the relative amounts of mRNA should be considered. The 11β-HSD1 mRNA in the DG and GR are low but relatively high in the CA3. The GR mRNA is high in the CA1, but relatively low in the other regions. However the MR mRNA is equivalent in each region. In the CA1, GR may dominate MR, but they are more equal in the CA3 and DG. The high level of 11β-HSD1 in the CA3, may mean that in the 11β-HSD1 knock-out (at nadir and intermediate levels of corticosterone) there is a particular increase in MR over GR signalling. In the other regions, this would depend upon any paracrine effects (due to overflow of locally produced corticosterone by neighbouring cells), but would perhaps be less significant.

The results of these in-situ-hybridisations in hippocampus are in contrast to an earlier report looking at mRNA in hippocampal sections in the 11β-HSD1 knock-out mouse on an MF1/129 background. This reported no change in hippocampal GR or MR mRNA (Harris et al. 2001). It is possible that there is a strain difference, although the plasma corticosterone profile is similar. It should be considered that the mice used in this earlier study were naïve and those from the current study had been previously used in Morris water-maze and Porsolt Forced Swim analysis (both of which are stressful tests). It may be possible that the effects seen incorporate some post-training / stress effects (Kitraki et al. 1999) and it would have been useful to include some mice which had not been behaviourally tested (as negative controls).

11β-HSD1 mRNA in the cerebellum

In many aspects, the results from the cerebellum stand alone. There have been few studies into glucocorticoids in the cerebellum and fewer still into glucocorticoid-mediated ageing.

There was no age-related decrease in the Purkinje neurons or the granular layer of any of the cerebellar lobules examined. This was in agreement with the results from the hippocampus. There was no age-related decrease in 11β-HSD1 or GR mRNA of the cerebellum. There have been no previous studies looking into this with in-situ-hybridisation.

Glucocorticoid receptor mRNA in the cerebellum

There were no changes in the GR mRNA of the Purkinje neurons. However, there was a pattern of reduced GR mRNA in the granular layer of the 11β-HSD1 knock-

out. This mirrors the decrease in GR mRNA seen in the CA1 region of the hippocampus. Such a decrease in the vermal region is of interest because this region is behaviourally linked with the hippocampus (reviewed in Lalonde 1997). It is also of interest that the greatest change in GR mRNA was seen in the granular region which expressed the least 11β-HSD1 mRNA. This suggests that the change is not due to a local change in 11β-HSD1 and could be interpreted as being secondary to a rise in plasma glucocorticoid. There has been just one reported study looking at glucocorticoid regulation within the cerebellum, this found a decrease in cerebellar GR mRNA in response to stress (Kitraki et al. 1999). There is not enough information available currently to confidently explain changes in GR mRNA in this region.

In contrast to the hippocampus, there was no significant change in GR mRNA with ageing. There have been no age-related changes described previously.

Implications for behaviour

How do these results correlate with the results found in the Morris watermaze, performed in these same animals (Yau et al. 2001)? There was no effect of learning found for genotype in the young. Nevertheless, the aged wild-type were found to be impaired and although the aged knock-out were not equivalent to the young, they were protected to a great extent compared to their age-matched controls. Thus the decrease of GR mRNA in CA1 does not seem to have had a significant effect upon maze learning in the young. However, it is possible that there was a floor effect in this test and any positive effects in the 11β-HSD1 knock-out group were not revealed. Pharmacological blocking of GR has previously improved spatial

learning (Oitzl et al. 1998b), but genetic disruption of the receptor has impaired learning (Oitzl et al. 2001; Rousse et al. 1997; Steckler et al. 1999). It was anticipated that absence of 11β-HSD1 would result in an improvement in learning.

As discussed in the introduction, the cerebellum is thought to be involved in, at least, the procedural or implicit aspects of watermaze performance (Mandolesi et al. 2001). There is very good evidence that the cerebellum is important in various classical-conditioning paradigms and is thought to be generally important in linking context with motor learning (reviewed in Woodruff-Pak 1997).

There remains a question as to why the aged knock-out show improved performance in the Morris watermaze. This could have been due to a change in MR : GR signalling pattern at the time of the test (Oitzl and de Kloet 1992; reviewed in Oitzl et al. 1997) or a progressive effect of long-term changes in local glucocorticoid.

2.4. Summary

This chapter sought to examine molecular aspects glucocorticoid signalling in the hippocampi and cerebella as a function of ageing and a life-time absence of 11β-HSD1. The main, novel finding of this study was that there was not a change in 11β-HSD1 mRNA in the hippocampal examined in aged male 129/Ola wild-type mice compared with young controls. This was despite an age-related spatial-learning deficit. Thus, the hypotheses that an increase in 11β-HSD1 mRNA with ageing contributed to corticosterone-related damage (by increasing local regeneration of corticosterone) was not upheld. In addition, there was no evident decrease in 11β-

HSD1 mRNA which could contribute to reduced glucocorticoid signalling. There was a significant decrease in GR mRNA in the CA1 region of the hippocampi of aged wild-type mice relative to their young controls. A similar significant decrease in CA1 was seen in the young 11β-HSD1 knock-out mice relative to the young wild-type mice, but an age-related change was not seen in the knock-outs. No significant differences were seen in MR mRNA between any groups. The results suggested that in the young 11β-HSD1 knock-out mice there was a change in local or peripheral glucocorticoid which reduced GR mRNA in the CA1 region. This was despite the relatively low levels of 11β-HSD1 mRNA in this sub-region. This upheld the hypothesis that reduced GR signalling in CA1, throughout life, had contributed to a local protection in ageing.

There was no change in 11β-HSD1 mRNA in the cerebellar regions examined in aged male 129/Ola wild-type mice compared with young controls. Equally, there were no age-related changes in GR mRNA between wild-type or knock-out groups in either the Purkinje neurons or granular neurons. There were no changes between groups in GR mRNA in the Purkinje neurons examined but some lobules. There was however, a significant reduction in GR mRNA with absence of 11β-HSD1 in the granular layers of some cerebellar lobules of both age groups. Like the hippocampal region, the reduction in GR mRNA did not co-localise with regions which expressed very high 11β-HSD1 mRNA in the wild-type. Exploration of glucocorticoid signalling in ageing is relatively sparse and this study should contribute to an understanding.

Future studies which look at: higher numbers; control for peripheral changes in glucocorticoid; and examine glucocorticoid aspects at the time of behavioural testing, thus facilitating correlations with behaviour, will help to clarify these results.

Chapter 3

Exploring Learning and Emotion in Young and Middle-Aged 11β-HSD1 Knockout Mice

3.1. Introduction

3.1.1. The Importance of Strain Background When Testing Phenotypes

When exploring animal models at the molecular, physiological or the cognitive level, it is essential to test on several genetic backgrounds. A gene modification may interact with background to create a different phenotype, thus reducing confidence in extrapolations to other species. There is much interest in the choice of background for testing genetic modification and pharmacological agent effects upon anxiety. A strain of mouse may have different levels of anxiety in different environments or use different coping strategies (Contet et al. 2001; Carola et al. 2002; Belzung et al. 2001).

We have previously shown in 129/Ola mice (described in Chapter 2), that 11β-HSD1 contributes to age-related impairments in a hippocampal dependent task (Yau et al. 2001). The attenuation of learning impairments in the aged 11β-HSD1 knock-out mice was hypothesised to be mediated through decreased local (cellular) corticosterone in sensitive neurons of the hippocampus (despite increased plasma corticosterone). As discussed in Chapter 1, there is a wealth of evidence supporting a role for dysregulated corticosterone levels in the development of hippocampal deficits with ageing in both rodents and humans (Yau et al. 1995; Issa et al. 1990; Lupien and Meaney 1998). The 129/Ola mice which had been tested previously (Yau et al. 2001), had demonstrated inadequate eye-sight for the classic Morris, watermaze spatial learning task (described below) and were tested in a visual platform variation, which had been shown to be hippocampal dependent. It was decided to test the hippocampal-protected phenotype on a C57BL/6J background.

This strain has been used in spatial learning tasks previously and exhibits intermediate anxiety (Montkowski et al. 1997; Calhoun et al. 1998; Frisch et al. 2000; Kawashima and Kusnecov 2002). To test whether spatial memory and other glucocorticoid associated behaviours are affected by the absence of 11β-HSD1 throughout life, the 11β-HSD1 knock-out mice were back-crossed (by Dr J. Patterson) for 10 generations onto a congenic C57BL/6J background.

3.1.2. Behavioural Testing

Spatial learning

Morris water-maze - In the current study, a 'Y-maze' was used to test spatial learning. However, the 'Morris water-maze' (introduced in Chapter 1) deserves some introduction here, if only to highlight aspects of spatial learning shared by the two mazes. The water-maze is a circular pool of water (Morris 1984), which is deep enough to force the rodent to swim. The pool is set within a room and for the animal to establish its position within the pool it needs to form connections between cues in the room. These cues should be varied, visible to the animal and not so unsubtle as to oversimplify the task. Global cues (e.g. a whole wall which is darkened) and proximal cues (e.g. a flag close to the target) probably do not require that different and plastic associations be made as the animal navigates its way through the pool. Such cues could be used by the entorhinal cortex to learn a task.

The classic testing paradigm is to submerge a platform just below the surface of opaque water. As the rats and mice do not 'enjoy' swimming, there should be a drive to find the platform (discovered accidentally in the learning trials) and escape the

water. The platform position remains constant but the rat is given several trials per day, to discover the platform, from different start points around the pool perimeter. Thus, over a period of days, a healthy rat or mouse should be able to learn the best combination of cues (perhaps using parallax etc) which will guide it to the platform. The ability of the hippocampus to maintain the integrity of combined stimuli, means that the animal can interpolate between overlapping sets of cues and maximise learning efficiency.

The previous study, examining spatial learning in aged 11β-HSD1 129/Ola knockouts (Yau et al. 2001) had used a visible platform variation of the classic Morris watermaze. This involved the use of a proximal visual cue for the platform, in addition to visual cues at the periphery of the pool. For those mice, this test was shown to be hippocampal-dependent by the use of hippocampal lesioned mice.

The Y-maze - The Y-maze was a new test for the laboratory, which required less training than the water maze and could be used to look at temporal (phasic) aspects of learning (e.g. acquisition or consolidation phases). This maze relies upon the positive drive of the rodent to explore a novel environment and is described later in this chapter. The Y-maze has previously been used to investigate the roles of MR and GR in spatial learning with great success (Conrad and McEwen 1997; Conrad et al. 1999) and is considered to be a more ethological learning paradigm than the watermaze. The Y-maze was also selected as a spatial learning test because it should be less stressful to older mice than the watermaze and did not require the food-restriction of other tests. Its introduction to the laboratory would also complement use of the watermaze.

Anxiety-related behaviours

In addition, considering the role played by glucocorticoids in anxiety (refer to Chapter 1) and possible relationships between this, depression and poor cognitive ageing, anxiety-related behaviours were examined. This would be tested using the elevated plus maze and the open-field. These are both common tests of unconditioned anxiety in rodents.

Motor co-ordination and learning

Little work has been done to explore age-related or glucocorticoid-related changes in cerebellar function. Glucocorticoids in the flocculus (a cortical cerebellar region closely associated with the vestibular system) had been shown to potentiate a form of compensation (Johnston et al. 2002). The accelerating Rota-rod was used to examine motor co-ordination and learning. This learning test has previously shown impairments in aged mice (Caston et al. 1998) and a simpler version has correlated oxidative damage in the cerebellum with performance (Forster and Sohal 1996).

Chapter outline

The chapter will progress to describe the general methods used (section 3.2) and then to describe the experiment with a discussion of results (section 3.3). Hypotheses were based upon the core hypothesis that 11β-HSD1 was acting as an 11-keto-glucocorticoid reductase (reactivating inert glucocorticoid) in the cerebellum). It was hypothesised that the middle-aged C57BL/6J control mice would show impaired spatial learning relative to the young controls and that the learning ability of the middle-aged 11β-HSD1 knock-out mice would be protected from this impairment. It

was also thought that the young and middle-aged 11β-HSD1 knock-out mice would demonstrate reduced anxiety behaviours relative to their age-matched C57BL/6J controls; such as increased open arm exploration in the elevated-plus-maze and decreased thigmotaxis in the open-field. Motor-learning was tested using the Rota-rod. In the motor-learning test, it was expected that an age-related deficit would be seen in the middle-aged C57BL/6J control mice relative to young controls. If that deficit were related to local glucocorticoid levels, there may be a protection of learning seen in the middle-aged 11β-HSD1 knock-outs. It was possible that a deficit would be seen in the young 11β-HSD1 knock-outs (and perhaps the middle-aged), if motor learning were dependent upon glucocorticoids - as demonstrated for another form of plasticity (Johnston et al. 2002). Based upon previous examination of the HPA axis in young 11β-HSD1 knock-out MF1/129 mice (Harris et al. 2001) and 129/Ola mice (Yau et al. 2001), it was hypothesised that an elevation of morning, unstressed levels and stressed levels of plasma corticosterone would be seen in young 11β-HSD1 knock-out mice relative to the young controls. Such findings would confirm the stability of the HPA axis phenotype upon a C57BL/6J genetic background and contribute to an understanding of behavioural findings.

3.2. General Methods

3.2.1. Experimental Animals

The 11β-HSD1 knock-out mice were generated from the 129/Ola 11β-HSD1 knock-out (Kotelevtsev et al. 1997) back-crossed onto C57BL/6 for 10 generations (Dr J. Patterson). Control male C57BL/6J mice were purchased from Harlan UK and maintained and aged in the same conditions. Animals were housed in groups of 3 to 5, food and water given ad-libitum and were maintained in a 12 hr light/dark cycle (lights on at 07:00 hrs). Each genotype was tested at middle-age (12 months) and young (3 months), (n=12 for each group). One young 11β-HSD1 knock-out and an aged control died during the course of testing.

3.2.2. Behavioral Testing

General conditions

All behavioural testing was performed between 09:00 and 12:00 hrs. Testing was performed in the AM phase to coincide with the HPA axis diurnal trough and thus to reduce masking of stress effects by high basal corticosterone values. The precise time of testing was a matter of procedural convenience. Animals were habituated to the testing room and handled for a week before the experiment began and were habituated to the room and handling, 1 hour before each test. Ambient light was kept dim (values taken at 100 ISA) and background sound minimised. Animal testing order was randomised and all tests were video-taped. In all tests (except the Rota-rod) the experimenter sat out of view of the subject and observed via a monitor. There were at least 6 days left between tests.

The Y-maze

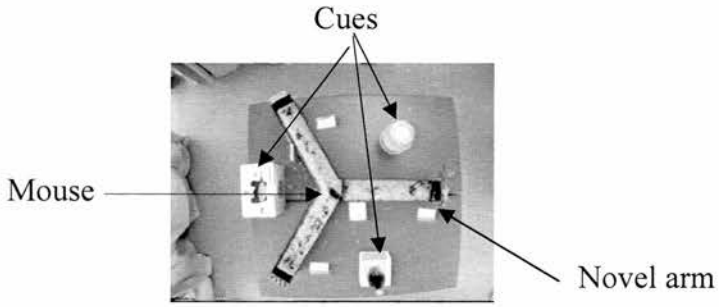

Figure 3-1: Experimental set-up for Y-maze spatial learning (acquisition trial)

The video still shows the Y-maze during the acquisition trial. The Novel arm was hidden from the mouse by a removable door and the mouse was allowed to explore the remaining arms for 5 minutes. The 3 dimensional visual cues were positioned so as to be visible to the mouse.

Response to novelty - The Y-maze was used to measure 'response to a novel arm' with 1 min and 2 hour delays and to measure 'spontaneous alternation' (in respective order) for each subject. Some weeks were spent establishing an appropriate room environment which would reveal positive and negative effects. The maze was constructed of grey ABS plastic (50 x 11 x 10 cm) (Figure 3-1) and the base covered in soiled sawdust which was stirred between trials and the walls were wiped with ethanol to hide scent trails. Each arm of the maze was assigned a geographical-name ('A', 'B', 'C') which remained constant throughout the experiment. A combination of proximal (black and white patterns behind the end-wall of each arm) and distal (objects from the lab e.g. clock) cues were arranged so that the mice would be able to see from the maze. The cues remained for the entire experiment.

In the 'response to a novel arm' test, each arm was assigned a test-name ('novel', 'other', 'start') for each animal-trial and the test-names were randomised between animals and tests. At the start of the 'exploration-trial' the 'novel' arm was blocked with a door (of the same material and height as the maze) and the mouse released at the distal end of the 'start' arm, facing the end-wall. The mouse was allowed to explore the open arms for 5 minutes. The mouse was then returned to the home-cage for the 'inter-trial-interval' (ITI) which was 1 or 120 minutes. Testing for 1 minute was intended to screen for the animal's ability to use the cues and short-term memory, whilst by 2 hours consolidation should have occurred and this should be a test of long-term memory. During the ITI the scent markers were disguised (sawdust mixed and wall wiped with ethanol) and the door was removed. At the start of the 'recognition-trial' the animal was placed in the 'start arm' as before and allowed to

explore for 5 minutes. Analysis was from video and the time of entry and exit for each arm was noted. As mentioned previously, animals were not re-tested before 6 days and this should be enough time for interference between tests to be avoided (Ladurelle et al. 2000). An arbitrary criterion of 40% exploration time in the novel arm was used to establish whether animals were preferentially exploring the 'novel' arm (Contarino et al. 1999).

In order to examine the memory retention after the 2 hour ITI and control for contaminating effects of variation in short-term memory, the exploration time after the longer and shorter it is were compared by Student-T test.

Spontaneous alternation - In the 'spontaneous alternation' test all arms were left open and the animal was introduced at the centre of the maze with the facing direction randomised. The animal was allowed to freely explore and the order of visits was noted, based upon the 'contextual-name' of the arm. Alternation is the visiting of 'alternate' arms within a trisomy, such that the maximum search efficiency is achieved for the energy expenditure of visiting arms. At the end of the 5 minutes, samples of blood were taken (refer above) to provide an indication of the HPA axis activity at the end of 5 minutes exposure to the maze.

The elevated-plus-maze

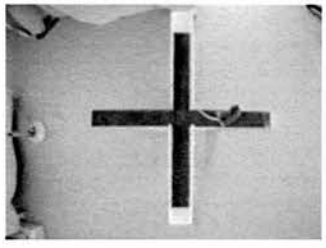

Figure 3-2: Experimental set-up for elevated-plus-maze

The video-still shows an arial view of the elevated-plus-maze apparatus. A mouse can be seen on an open arm. Two of the arms were enclosed by white transluscent plastic and two were open and exposed the mouse to the 1m elevation of the platform. Aspects of exploration of the open and closed arm could be used to analyse anxiety and exploratory behaviour.

The floor of the elevated-plus-maze (25 x 6 cm) was made from black, opaque acrylic and the surface was dulled using fine sandpaper to minimise reflection of light. The walls (15 cm high) were constructed from a pearl, translucent acrylic (5mm thick) (Figure 3-2). This meant that the lighting contrast between open and closed arms was minimised (exposure values 3.2 vs 2.6) and the maze was based upon exposure and height. The maze was supported above the ground at 1m, height cues were minimised. Animals were placed centrally with the head facing an open arm and allowed to explore for 5 minutes. The maze floor and walls were cleaned with alcohol between tests. Analysis was done from videotape, recording arm entry number and time and more ethological measures such as head dips (looking over the open edge with the head out), open-protected-stretches (the forelimbs stretched onto the open arm with the rear in the closed or centre), closed-protected-stretches

(stretched exploration with the whole body in the closed area), number of faeces and any urination.

The open-field

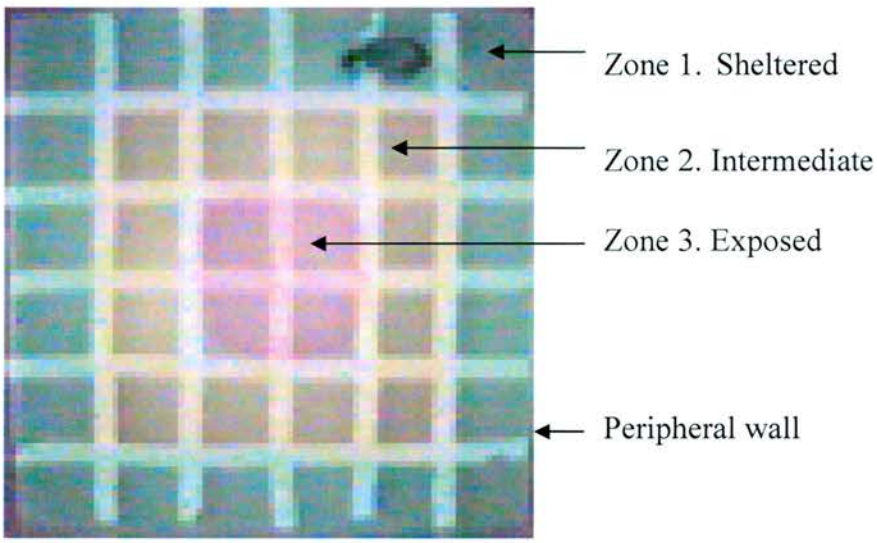

Figure 3-3: Experimental set-up for the open-field, free exploration

The video-still shows an arial view of the open-field apparatus with a mouse in the Zone 1. In this apparatus, the opaque wall (15cm high) acted as a shelter to the animal. Exploration of this zone and the more exposed zones was analysed by measuring the locomotion within each zone (colours are for reference only). This was done manually by counting crossings between the smaller squares (marked with white tape). Ethological measures were also made.

Lighting was aimed at the central zone whilst minimising uneven shadows and reflection points, such that the exposure value in the outer zone was 4.0 and central zone was 4.6. The subject was placed in a corner square, facing the corner, and observed for 300s. Values measured included time to central zone, square crossings in each zone (crossings of all 4 legs), time spent in non-motile (to measure freezing behaviour and as in inverse correlate of time spent motile), grooming sessions, vertical exploration (rearing against the wall) and rearings (unsupported).

The Rota-rod

The 'Accelerating Rota-rod treadmill for mice' (cat. 7650) was obtained from Ugo Basile, Biological Research Apparatus. Each section (there were 5 on this rod, although 1 was used) measured 5cm and had a 'knurled' surface for grip. When testing for motor-learning the Roto-rod gearing was set to the highest point to obtain acceleration from 4rpm to 40rpm in 5 minutes. Mice were tested one at a time. The mouse was held by the tail and set on the rotating rod (4rpm) against the direction of rotation. The operator sat behind the mouse. The rod was maintained at 4rpm for 10 seconds. A mouse which stayed on this for 10 seconds (arbitrary value) had reached criterion and the rod was allowed to accelerate, the time until the mouse dropped was recorded. If the mouse fell off within the 10 seconds, it was reapplied. A mouse was allowed to make 10 such attempts and the number of failed attempts was recorded. This was repeated for a total of 5 days and the values plotted as a learning curve.

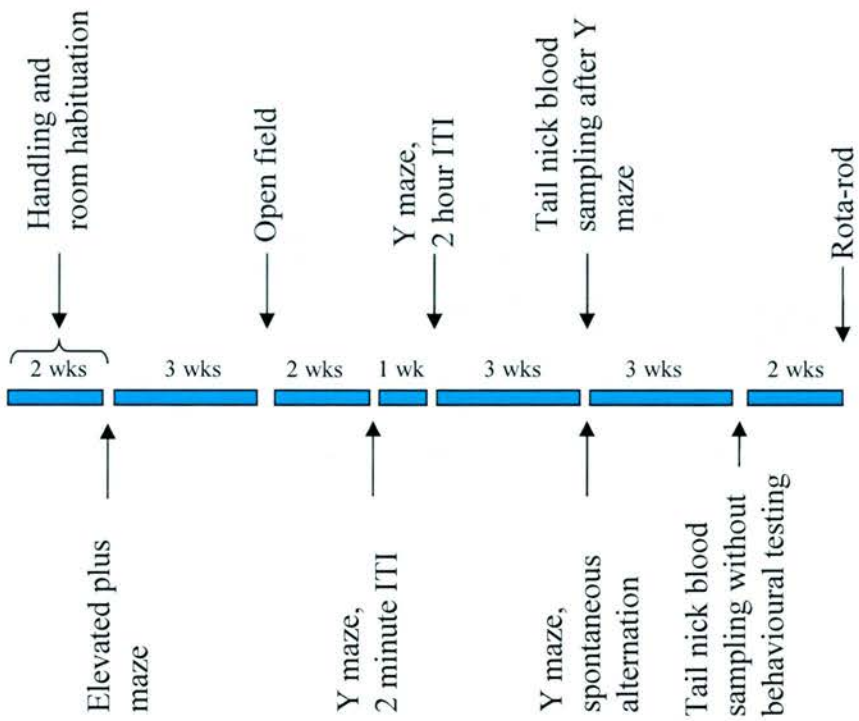

Figure 3-4: Summary of experimental schedule

The cartoon shows the schedule of behavioural testing and blood sampling for plasma corticosterone samples. An initial period of handling and habituation was followed by anxiety and exploration testing in the elevated-plus maze and the open field. Spatial learning was then tested in the Y-maze using various inter-trial-intervals (ITI), exploration of the Y-maze was tested with spontaneous alternation. Blood samples were taken directly after Y-maze spontaneous exploration testing and at the same time of day (to control for diurnal variation) to control for the stress associated with Y-maze exploration. Motor learning was tested using the Rota-rod.

3.2.3. Measurement of Plasma Corticosterone

Sampling

Samples were taken from tail nicks directly after the 'spontaneous-alternation, Y-maze' test. Mice were habituated to the technique for some days before and samples

were taken in a room adjacent to the test room. Animals were allowed to recover from the sampling and some weeks later a base-line sample was taken at the same time of day.

The mouse was allowed to rest on the cage lid and the tail was nicked with a razor. Samples were taken using Microvette tubes and stored on ice until centrifuged at 4°C. The samples were stored at -20°C until use.

Corticosterone assay

All samples were tested simultaneously by radio-immunassay (MacPhee et al. 1989), modified for microtiter plate scintillation proximity assay. Samples were diluted (to 10%) in borate buffer (135nM sodium borate (pH 7.4), 0.5% bovine serum albumin, 1% methanol, 0.1% ethylene glycol) and heated to 70°C (1 hour) to inactivate plasma, binding proteins. ^{3}H-corticosterone (s.a. 84 Ci/mmol) and rabbit antirat-corticosterone antiserum (Dr. C. Kenyon) (final antibody dilution 1:10000 in borate buffer containing 0.01% Na azide) were added to each sample and incubated at room temperature (1 hour). A secondary antibody, bound to fluomicrospheres (anti-rabbit SPA reagent, Amersham) was added. The radio-labelled antibody bound to the secondary antibody is immobilised by the fluomicrospheres, which then emit light which was detected on a β-scintillation counter 24 hours later. Samples were assayed in duplicate and their corticosterone concentration assayed against a serial dilution of corticosterone. The intra-assay coefficient was 9.4% and the inter-assay was 9.2%.

3.2.4. Statistics

Data are presented as mean values per group ± s.e.m. Data from all four groups were compared by 2-way ANOVA with age and 11β-HSD1 genotype as independent variables. This was followed by a Fisher LSD-post hoc test, where appropriate. Frequency distribution was analysed by the Chi squared test (χ^2). Significance was set at p=0.05.

3.3. Results

3.3.1. Behaviour

The Y-maze

Response to novelty - Results for each 1 minute time-bin of the recognition trial were collected, and interpreted for the first 2 minutes, the latter 3 minutes and the full 5 minutes. 1 young control mouse did not leave the 'start' arm during the 2 hour ITI recognition trial and was excluded from that analysis.

Time spent in each arm was calculated as a percentage of the time spent exploring the 'arms'. After the 1 minute ITI, all groups spent more exploration time in the 'novel' arm (Figure 3-5) than in the 'other' or 'start' arms. When the time spent in the 'novel' arm was compared with the time spent in the 'start' arm (Student-T test) there were significant differences seen in the young controls ($T_{(12,20)}$=6.64, $p<0.01$), young 11β-HSD1 knock-outs ($T_{(11,20)}$=6.26, $p<0.01$), middle-aged controls ($T_{(11,20)}$=5.06, $p<0.01$) and middle-aged 11β-HSD1 knock-outs ($T_{(22.12)}$=7.79, $p<0.01$). This difference was concentrated within the first 2 minutes of exploration. However, after the 1 minute ITI, there was no difference in the percentage exploration time spent in the 'novel' arm between groups (ANOVA).

After the 2 hour ITI, the middle-aged 11β-HSD1 knock-out showed significantly improved memory retention (percentage time in the 'novel' arm) compared with their age-matched controls [ANOVA revealed a significant effect of genotype (Figure 3-6) ((first 2 minutes ($F_{(1,41)}$=8.60 , $p<0.005$); (over the 5 minutes ($F_{(1,41)}$=5.03 , $p<0.03$))].

In addition, a comparison of the percentage time spent in the 'novel' arm compared with the 'start' arm showed that only the knock-out groups spent significantly more time in the 'novel' arm: young controls ($T_{(12,20)}$=1.13, p=0.27); young 11β-HSD1 knock-outs ($T_{(11,20)}$=2.83, p<0.01); middle-aged controls ($T_{(11,20)}$=0.38, p=0.71); and middle-aged 11β-HSD1 knock-outs ($T_{(22.12)}$=5.38, p<0.01).

There were no differences between the groups in the numbers of mice reaching criterion (40% time in the novel arm) (Figure 3-7): young controls vs middle-aged controls (χ^2=1.05, p=.31); young controls vs young knock-outs (χ^2=0, p=1.0); young knock-outs vs middle-aged knock-outs (χ^2=0, p=.94); middle-aged controls vs middle-aged knock-outs (χ^2=0, p=1.0). It should be noted that the number of animals reaching criterion was reduced in all groups after the longer interval but that fewer of the middle-aged 11β-HSD1 knock-out achieved criterion: young controls vs middle-aged controls (χ^2=3.14, p=.08); young controls vs young knock-outs (χ^2=0, p=1.0); young knock-outs vs middle-aged knock-outs (χ^2=1.06, p=0.30); middle-aged controls vs middle-aged knock-outs (χ^2=7.43, p<0.01). The response-to-novelty was maintained in the 11β-HSD1 knock-outs, with no obvious differences between their 1 minute ITI and 2 hour ITI scores (compare figures 3-5 and 3-6). Indeed, a T-test comparison of the percentage time in the 'novel' arm after the 1 minute ITI and 2 hour ITI, demonstrated significant reductions in the C57BL/6J control groups but not in the 11β-HSD1 knock-out groups (Table 3-1).

There were no factor differences in activity found in the number of visits to arms (ANOVA) (Figure 3-8). However, there were differences found at LSD post-hoc. There was an age-related decrease in activity in all arms of the maze in the control

C57BL/6J mice, over the five minutes of exploration. This was concentrated in the 'start' arm in the first 2 minutes ($p<0.05$) and in the 'novel' arm ($p<0.05$) over the latter 3 minutes, and was significant when overall activity (entries into all arms) was examined in both time bins. In addition, the middle-aged 11β-HSD1 knock-out mice made more overall entries and specifically in the 'novel' arm in the latter 3 minutes than their age-matched controls ($p<0.05$). The only significant difference between groups after the 2 hour ITI, was an increase in 'novel' arm entries ($p<0.05$) by the middle-aged 11β-HSD1 knock-out mice relative to their age-matched controls in the first 2 minutes of exploration. When the number of entries to arms was adjusted for overall activity (total visits to arms), all differences were lost (Figure 3-9).

Figure 3-5: Percentage exploration time spent in the arms of the Y-maze during the recognition trial after 1 minute I.T.I.

The upper chart shows the percentage exploration by the groups in arms of the Y-maze over the 5 minutes of the recognition trial, to test short-term spatial memory. Mice were placed in the 'start' arm and allowed to explore this and the 'other' arm for 5 minutes for the exploration trial. The mouse was then removed from the maze for a 1 minute –inter-trial-interval (ITI) and re-introduced to freely explore the 'start', 'other' and 'novel' arms. There were no significant differences between groups. The charts do show preferential exploration of the 'novel' arm.

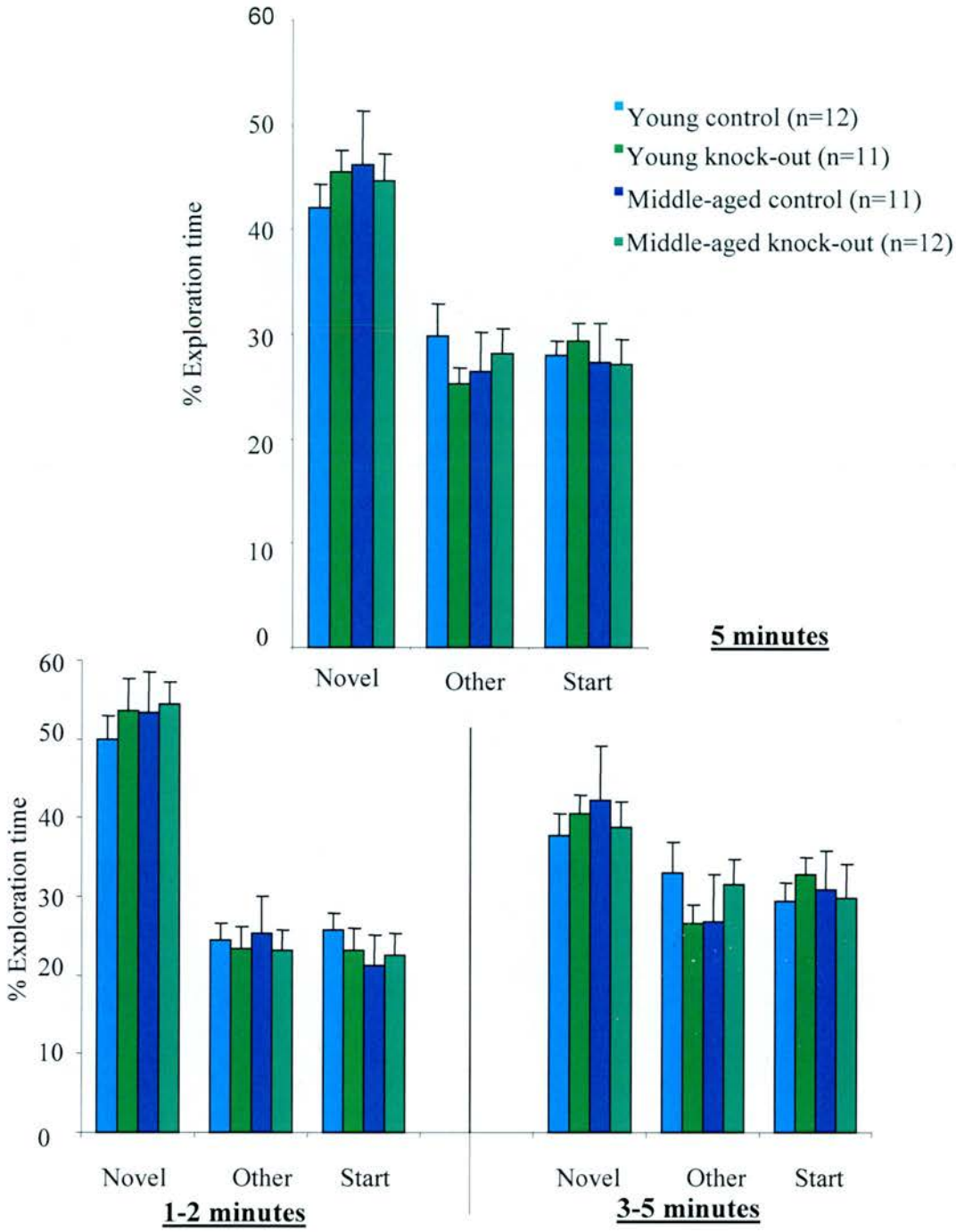

Figure 3-5: Percentage exploration time spent in the arms of the Y-maze during the recognition trial after 1 minute I.T.I.

Figure 3-6: Percentage exploration time spent in the arms of the Y-maze during the recognition trial after 2 hours I.T.I.

The upper chart shows the percentage exploration by the groups in arms of the Y-maze over the 5 minutes of the retrieval trial, to test long-term spatial memory. The middle-aged 11β-HSD1 knock-out showed increased percentage exploration of the novel arm compared with middle-aged controls over the full 5 minutes. This was concentrated in the first 2 minutes, * = $p<0.05$ LSD post-hoc analysis.

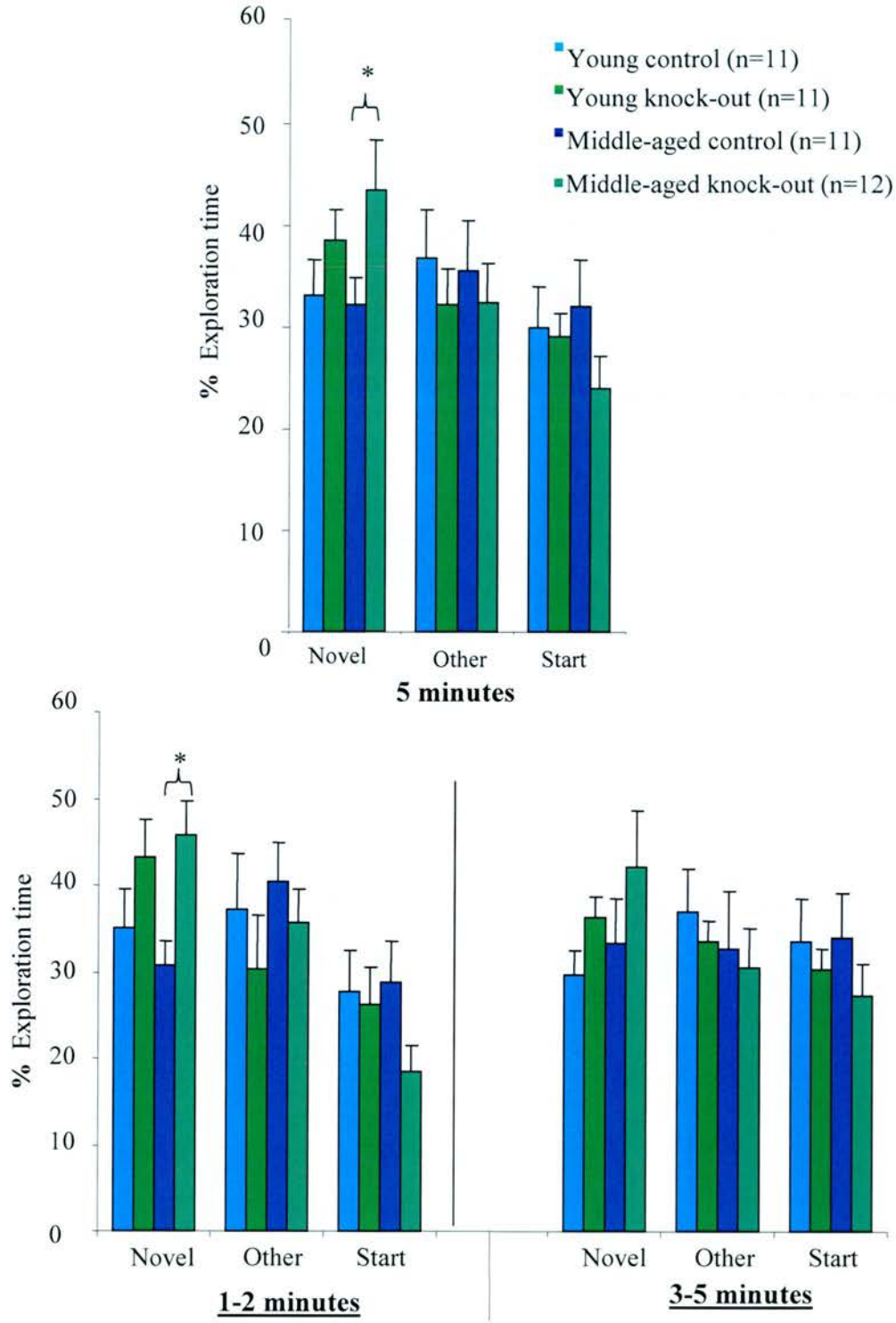

Figure 3-6: Percentage exploration time spent in the arms of the Y-maze during the recognition trial after 2 hours I.T.I.

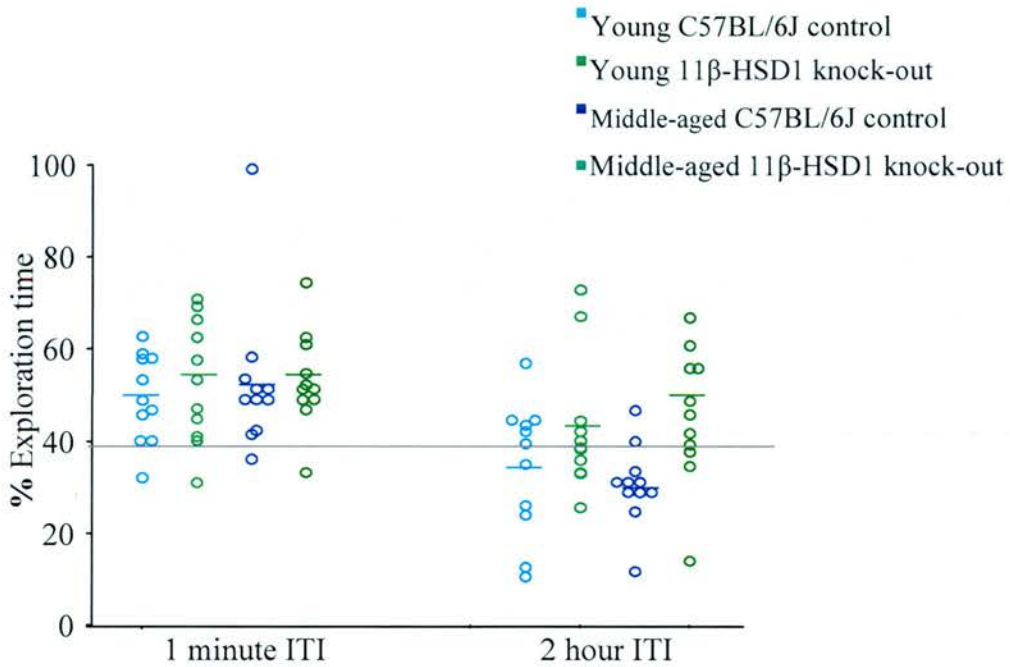

Figure 3-7: Percentage exploration time (first 2 minutes) of the novel arm in the recognition trial of spatial learning, after a short and long inter-trial-interval

The chart represents data shown in figures 3-5 and 3-6, with each circle representing a separate animal. The middle-aged knock-outs are more likely to reach 40% criterion after a 2 hour inter-trial-interval than their C57BL/6J controls. When looking at the numbers reaching criterion: there were no differences in the short ITI, but fewer of the middle-aged controls reached 40% than the middle-aged 11β-HSD1 knock-outs (X^2=7.43; $p<0.01$).

Table 3-1: 'Consolidation' in the 2 hour I.T.I. Y-maze

Group	t	df	p	cases
Young C57BL/6J control	1.73	22	0.10	12
Young 11β-HSD1 knock-out	4.00	20	0.00	11
Middle-aged C57BL/6J control	1.74	20	0.10	11
Middle-aged 11β-HSD1 knock-out	2.81	20	0.01	11

T-test analysis of retrieval trial performances (% exploration time spent in the novel arm) after the 1 minute inter-trial interval (ITI) and the 2 hour ITI, for each group. The control groups showed significantly reduced performance after the longer ITI, whereas the knock-out groups showed no difference between performance. This suggested that the controls did not fully consolidate the memory demonstrated after 1 minute ITI, whereas the knock-outs did.

Figure 3-8: Number of visits to arms of the Y-maze (recognition trial), with a 1 minute and 2 hour inter-trial-interval.

The charts show the number of visits (all 4 paws in) to the arms of Y-maze during the recognition trial, after a 1 minute and a 2 hour inter-trial-interval (ITI). For each recognition trial, the full 5 minutes of exploration and partial analyses are shown.

1 minute I.T.I.: Over the full 5 minutes, the middle-aged controls were less active than the young controls in all arms of the maze. This was significant in the 'start' arm in the first 2 minutes and in the 'novel' arm in the latter 3 minutes. In the latter 3 minutes, the middle-aged knock-out made more visits to the 'novel' arm than the middle-aged controls.

2 hour I.T.I.: After the longer ITI, the only significant difference was in the first 2 minutes, when the middle-aged knock-outs made more visits to the novel arm than the middle-aged controls.

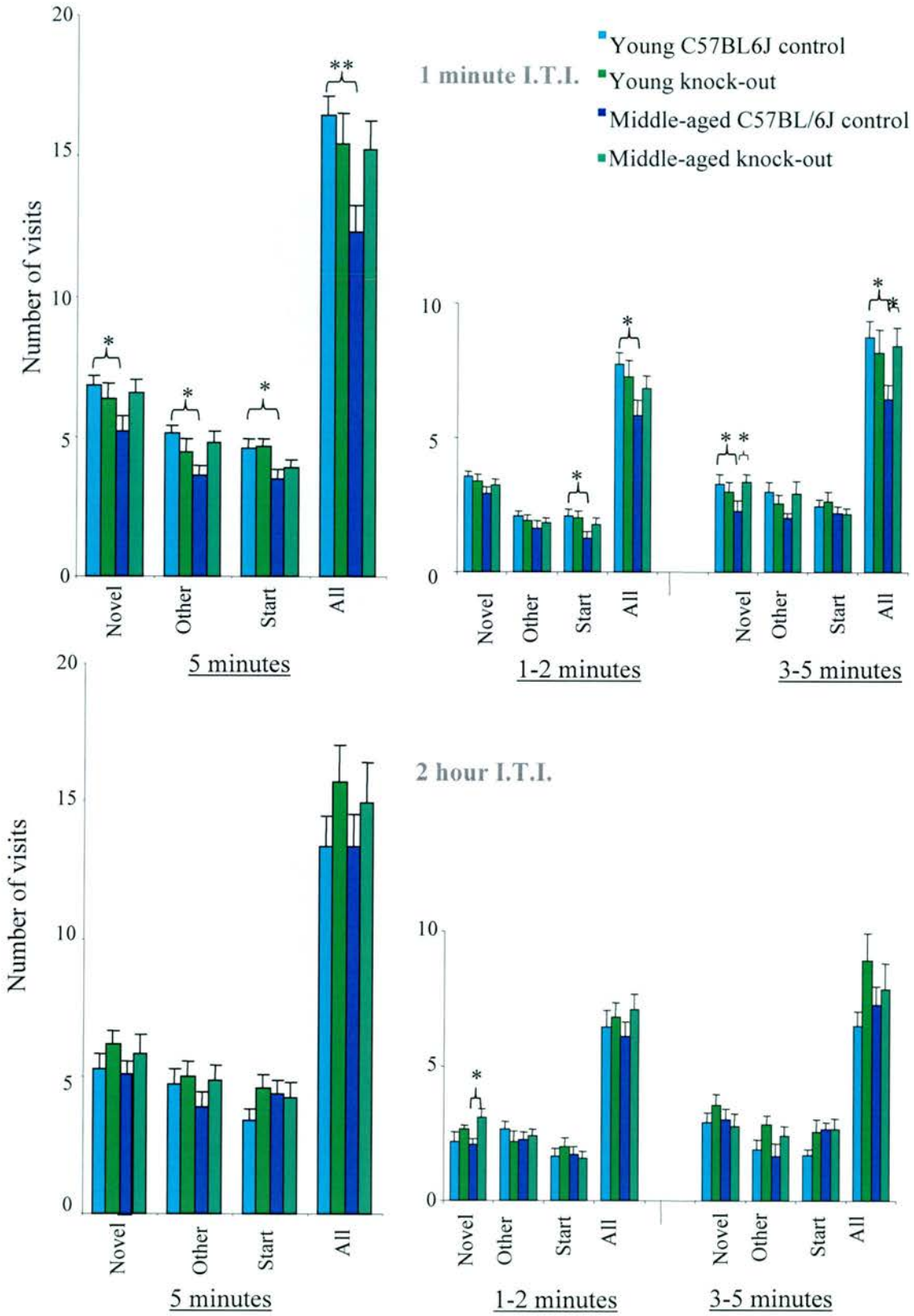

Figure 3-8: Number of visits to arms of the Y-maze (retrieval trial), with a 1 minute and 2 hour I.T.I.

Figure 3-9: Number of visits to arms of the Y-maze (recognition trial), as a percentage of overall visits

In order to examine arm preference in the Y-maze whilst controlling for overall motor activity, the number of visits by each mouse was divided by the total number of visits. All differences which had been seen when looking at absolute values (Figure 3-8) were lost.

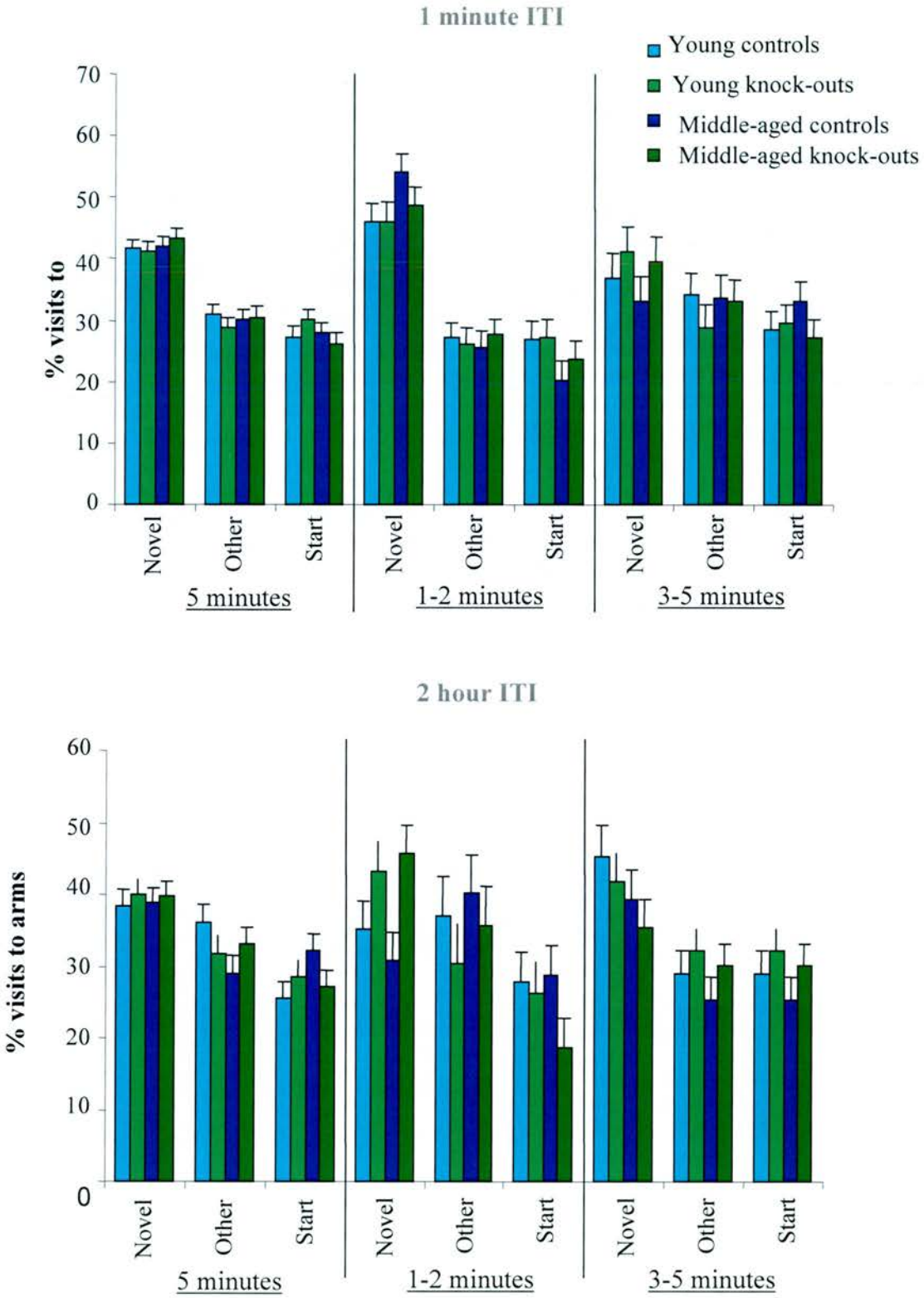

Figure 3-9: Number of visits to arms of the Y-maze (recognition trial), as a percentage of overall visits

When they left the 'start arm, the mice always entered the 'novel' or 'other' arm, never returning immediately to the 'start' arm (Table 3-2). After 1 minute ITI, there were no differences in the proportions of mice choosing the 'novel' arm (analysed by Chi-squared): young control vs middle-aged control (X^2=0.39; p=0.53); young control vs young knock-out (X^2=1.05; p=0.31); young knock-out vs middle-aged knock-out (X^2=0.0; p=1.0); middle-aged control vs middle-aged knock-out (X^2=2.94; p=0.09). There were also no differences after the 2 hour ITI: young control vs middle-aged control (X^2=2.94; p=0.09); young control vs young knock-out (X^2=0.21; p=0.65); young knock-out vs middle-aged knock-out (X^2=0.38; p=0.54); middle-aged control vs middle-aged knock-out (X^2=0.01; p=0.92). The latency to leave the start arm was measured to investigate any speed vs. accuracy compensations associated with the first-arm-choice (Table 3-3). There were no group differences after the 1 minute ITI, but after the 2 hour ITI, the middle-aged 11β-HSD1 knock-outs left the 'start' arm faster than the young 11β-HSD1 knock-outs.

Table 3-2: Percentage mice making the correct first-arm-choice in the recognition trial of the Y-maze spatial learning

Group	1 minute ITI	2 hour ITI
Young C57BL/6J control	**91%**	**58%**
Young 11β-HSD1 knock-out	**100%**	**71%**
Middle-aged C57BL/6J control	**83%**	**82%**
Middle-aged 11β-HSD1 knock-out	**82%**	**92%**

As mice first exited the 'start' arm during the retrieval trial, their choice of 'novel' or 'other' arm was recorded. The chance level of choosing the 'novel' arm was 50%. After 1 minute ITI and the 2 hour ITI, there were no differences in the group frequencies of mice choosing the 'novel' arm.

Table 3-3: Latency (seconds) to leave the 'Start' arm in the recognition trials of the Y-maze spatial learning test

Group	1 minute ITI	2 hour ITI
Young control C57BL/6J	13 ± 2	15 ± 2
Young 11β-HSD1 knock-out	15 ± 3	21 ± 4*
Middle-aged control C57BL/6J	15 ± 7	10 ± 2
Middle-aged 11β-HSD1 knock-out	7 ± 1	10 ± 1*

The time taken to initially leave the start arm was measured to establish whether there was a 'trade-off' between arm choice accuracy and the speed of decision making (group mean ± sem). At the 1 minute ITI, there were no differences between groups (ANOVA). After the 2 hour ITI, there was an effect of age ($F_{(1,42)}$=12.9, $p<0.001$) but not of genotype ($F_{(1,41)}$=1.4, $p=0.24$), with no interaction between the 2 factors. The middle-aged 11β-HSD1 knock-outs left the 'start' arm faster than the young knock-outs (LSD post-hoc) ($p<0.001$).

Spontaneous alternation - The series of arm entries were recorded and the degree of alternation was calculated. All groups achieved above the chance level of spontaneous alternation (22%) (Table 3-4). There were no significant differences between the groups. In addition, there were no differences in the total number of arm entries between the groups. There was no evidence of any geographical arm or order bias.

Table 3-4: Spontaneous alternation in the Y-maze

Group	% alternation	Number of arm entries
Young control C57BL6J	73.0 ± 5.7	20.8 ± 4.9
Young knock-out	65.1 ± 4.3	20.5 ± 5.0
Middle-aged control C57BL/6J	70.5 ± 6.6	18.5 ± 4.7
Middle-aged knock-out	72.7 ± 7.2	20.9 ± 6.0

The animals were allowed to freely explore the 3 arms of the maze for 5 minutes. The results show the percentage alternation over the 5 minutes (mean ± sem). There were no differences between the groups in the degree of spontaneous alternation (ANOVA) – an indicator of the ability to use allocentric navigation and working memory. There were also no differences in the number of arm entries (ANOVA) – an indicator of exploration activity.

The elevated-plus-maze

Arm entries - The plus maze appeared to be rather anxiogenic (Figure 3-10), with relatively few visits to the open arm. In the first 2 minute time-bin, the young 11β-HSD1 knock-out made more open arm entries than their young controls or the middle-aged 11β-HSD1 knock-out. Over the full five minutes the core difference between groups was between the middle-aged C57BL/6J controls and middle-aged 11β-HSD1 knock-outs, with the knock-outs making more closed arm entries (this difference was focussed on the latter 3 minutes of testing). By ANOVA, there were no effects of age, but the knock-out were more likely to make an entry into a closed arm in the later phase of exploration ($F_{(1/41)}= 6.8$, $p<0.01$).

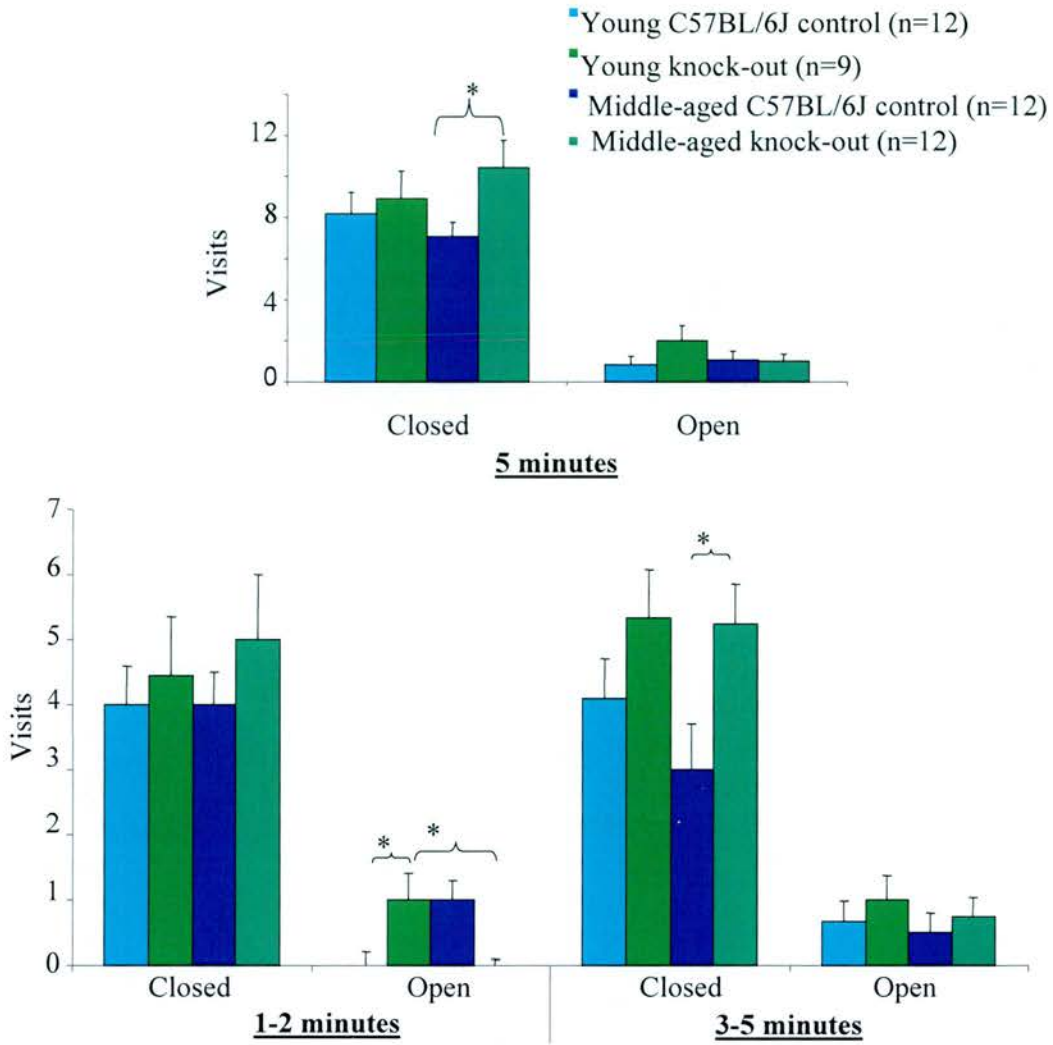

Figure 3-10: Visits to closed and open arms of the elevated-plus-maze

The upper chart shows the number of visits to the arms over the full 5 minutes of exploration. The lower charts show partial analysis. Results are shown for young controls, the young 11β-HSD1 knock-outs, the middle-aged controls and the middle-aged 11β-HSD1 knock-outs. Over the 5 minutes, the middle-aged 11β-HSD1 knock-outs make more closed arm entries than their middle-aged controls. In the first 2 minutes, the young 11β-HSD1 knock-outs made more open arm entries than the young controls, the middle-aged controls more than the middle-aged 11β-HSD1 knock-outs. In the latter 3 minutes, the middle-aged 11β-HSD1 knock-outs made more closed arm entries than the middle-aged controls. * = $p<$ 0.05 LSD post-hoc analysis.

Some studies have used proportion of time spent in the arms as a measure of anxiety. Over the full 5 minutes (Figure 3-11), there was a small, but significant increase in the time spent in the closed arms with age ($F_{(1,41)}=5.4$; $p<0.05$). This difference correlates with a reduction in time spent in the central section of the maze ($F_{(1,41)}=13.0$; $p<0.001$). There were no differences in the time spent in the open arms of the maze, which was very low.

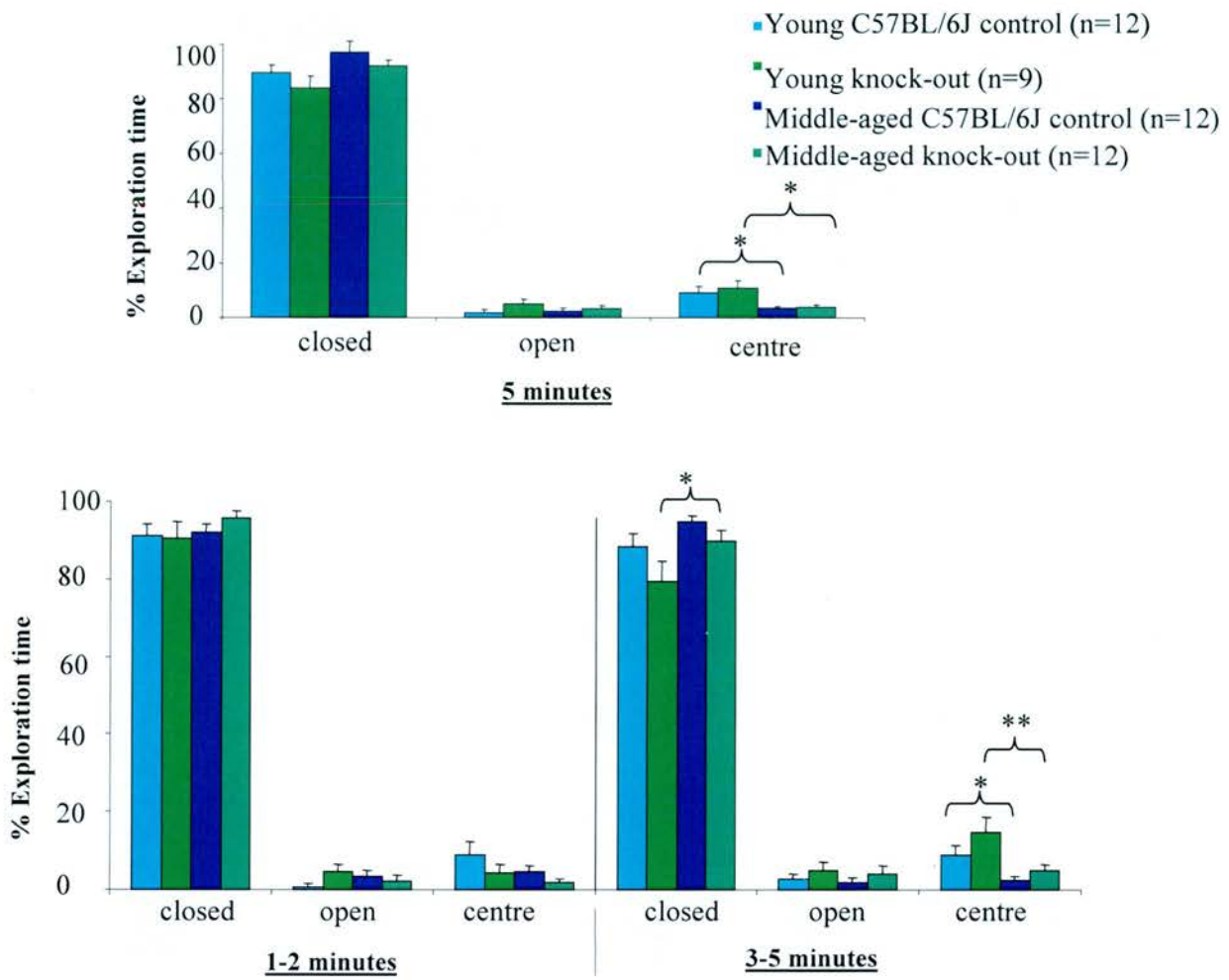

Figure 3-11: Percentage exploration time spent in areas of the elevated-plus-maze, over the full 5 minutes and showing partial analysis

The upper chart shows exploration over the full 5 minutes and lower charts show partial analysis into time bins. Over the 5 minutes, the middle-aged groups spent less time on the central platform than their younger genotype groups. In the first 2 minutes, there were no significant differences. In the latter 3 minutes of exploration, the middle-aged 11β-HSD1 knock-outs made spent more time in the closed arm than the young 11β-HSD1 knock-outs and less time on the central platform. The middle-aged controls spent less time on the central platform. *= $p< 0.05$, ** =$p<0.005$ LSD post-hoc analysis

Ethological measures - There were differences found in the ethological aspects of platform exploration. The young 11β-HSD1 knock-outs made more open-protected-stretches (OPS) than their age-matched controls over the full 5 minutes of exploration (Figure 3-12). This difference was not seen in the older mice. There was a difference in the pattern of OPS between the young and old, with the young focusing such risk-assessment in the later part of exploration, the middle-aged groups in the earlier. By ANOVA, there was an effect of 11β-HSD1 upon open-protected stretches over the 5 minutes ($F_{(1,41)}$=4.5; $p<0.04$), with no interaction between age and genotype ($F_{(1,41)}$=2.15; $p<0.15$). There were no differences in the open-unprotected-stretches or closed-protected-stretches made by any of the groups and the frequency approached zero over the 5 minute. There was an age-effect upon the number of head-dips in the first 2 minutes of exploration, but the young increased the number of dips in the latter time bin, with the young 11β-HSD1 knock-outs making significantly more dips than the middle-aged 11β-HSD1 knock-outs.

When such measures of exploration were analysed in early and late time bins, more effects were revealed. In the early phase the number of OPS and of head-dips increased with age in both genotypes. There was no effect of genotype. However, over the last 3 minutes, this exploration decreased with age and the young 11β-HSD1 knock-outs made more OPS than the young control C57BL/6Js.

Other ethological measures of behaviour were recorded. The number of grooming sessions recorded was consistently low across the 5 minutes, with no differences. The presence of faeces and urine were also recorded (Table 3-5). The young knock-outs were more likely to leave a faecal bolus than the young controls and the middle-

aged knock-outs were more likely to micturate than the middle-aged controls (Chi-squared).

Figure 3-12: Ethological measures of exploration in the elevated-plus-maze

The upper chart shows the number of times a behaviour was performed over the full 5 minutes of exploration. The lower chart shows partial analysis into 2 time bins. Over the full 5 minutes the 11β-HSD1 knock-outs made more open-protected-stretches (fore-paws on the open arm, hind-paws on the central platform). There were no differences in the open-unprotected stretch (a stretching exploration on the open arms), the closed-protected stretch (a stretching exploration on the closed arms) or in the head-dip (the head over the edge of the platform, exploring the area below). Open-unprotected-stretch and closed-protected stretches are not shown in the partial analysis because of the low numbers involved. In the first 2 minutes, the middle-aged groups, showed increased open-protected-stretches and head-dip compared with their younger genotype groups. Conversely, in the latter 3 minutes, the young groups showed more open-protected-stretches than their middle-aged genotype groups, and the young 11β-HSD1 knock-outs showed more than the young controls. There was an age-related change in the knock-outs in head-dips in the latter time bin, with young 11β-HSD1 knock-outs making more head-dips than the middle-aged knock-outs. *= $p< 0.05$, ** =$p<0.005$, *** =$p<0.0005$ LSD post-hoc analysis

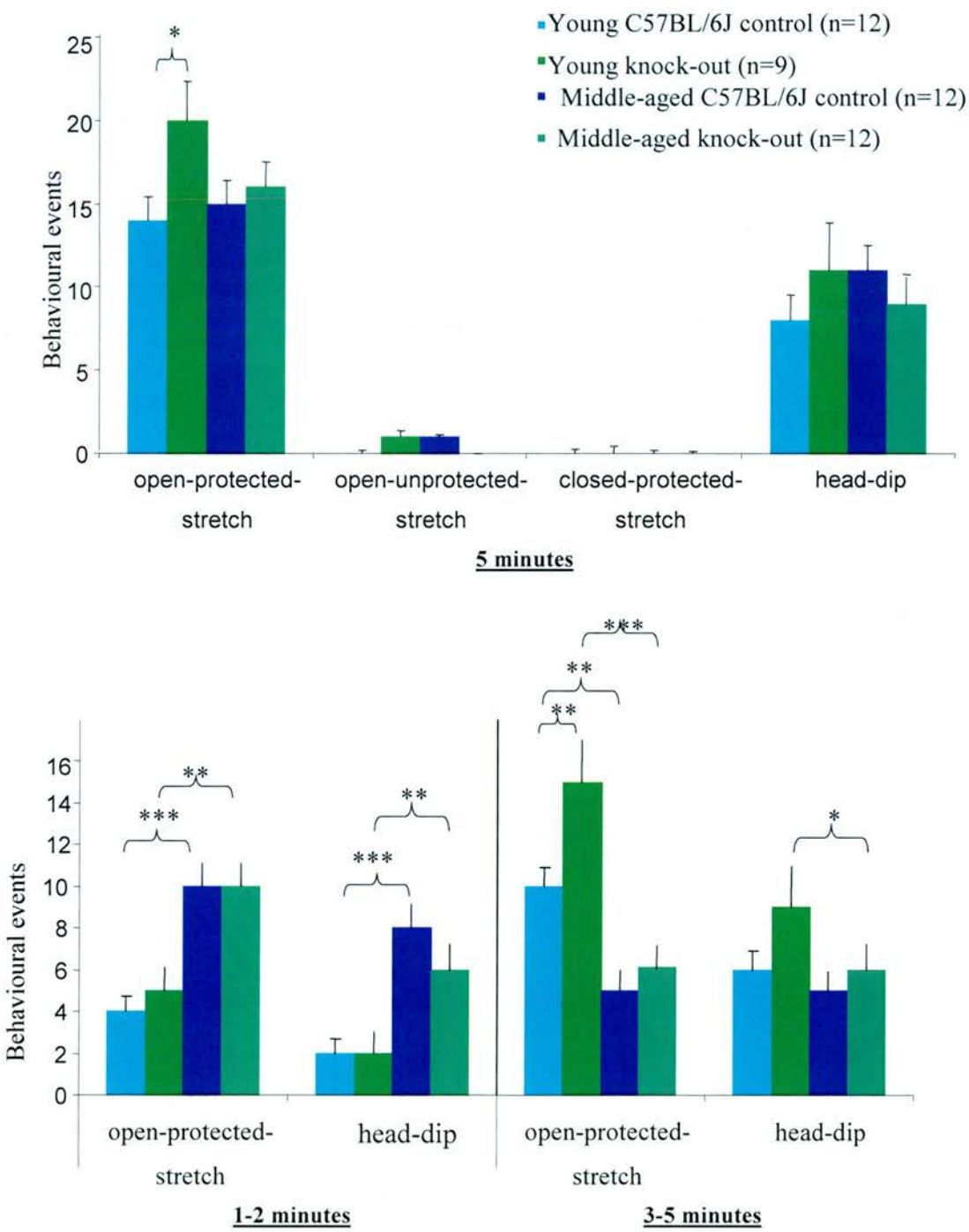

Figure 3-12: Ethological measures of exploration in the elevated-plus-maze

Table 3-5: Percentage of mice leaving faeces or urine during exploration of the elevated-plus-maze

Groups	**Faeces**	Urine
Young control C57BL/6Js	50%	25%
Young 11β-HSD1 knock-outs	67%	33%
Middle-aged control C57BL/6Js	58%	17%
Middle-aged 11β-HSD1 knock-outs	58%	33%

The results represent group frequencies of defaecation and micturation over the 5 minutes of exploration. No mouse left more than 1 faecal bolus. Analysed by chi-squared, the young knock-outs were more likely to leave a faecal bolus than the young controls ($p<0.05$). There were no other group differences. The middle-aged knock-out mice were more likely to micturate than the middle-aged controls ($p<0.01$). There were no other group differences.

The open-field

Square crossings - The young 11β-HSD1 knock-outs were more active than their young controls, making more total crossings (Figure 3-13) than the young control C57BL/6Js but there were no differences in zones 2 and 3. This difference was focused upon the first 2 minutes of exposure. When crossings in each of the zones was adjusted for overall activity (Figure 3-14) there was a positive effect of 11β-HSD1 absence in the outer zone (z1) ($F_{(1,44)} = 4.48$, $p<0.05$) and a negative effect in the intermediate zone (z2) ($F_{(1,44)} = 5.1$, $p<0.05$).

The young 11β-HSD1 knock-outs spent a lower proportion of the time non-motile (Table 3.6) than the young controls. By 2-way ANOVA there was no effect of age upon time spent immobile but the presence of the 11β-HSD1 gene had a positive effect upon immobility (90.3 vs 58.4, $F_{(1,44)} = 8.51$; $p<0.01$). Interaction between the 2 variables was significant ($F_{(1,44)} = 4.00$; $p=0.05$).

Figure 3-13: Square crossing in zones of the open-field-maze

Horizontal locomotion in the open-field, was measured by counting crossings (by all 4 paws) into sub-squares of the concentric zones of the maze floor (refer to Figure 3-4). The upper chart shows exploration over the full 5 minutes and lower charts show partial analysis. Over the 5 minutes, the young knock-outs show more activity (square crossings) in the outer zone (z1) than the young C57BL/6J controls. In the first 2 minutes, the middle-aged controls showed more activity than the young controls, overall and in z1 and z3. The young knock-out also showed more activity than the young wild-types, overall and in z1. In the latter time bin, there were no differences between groups. *= $p<0.05$ LSD post-hoc analysis.

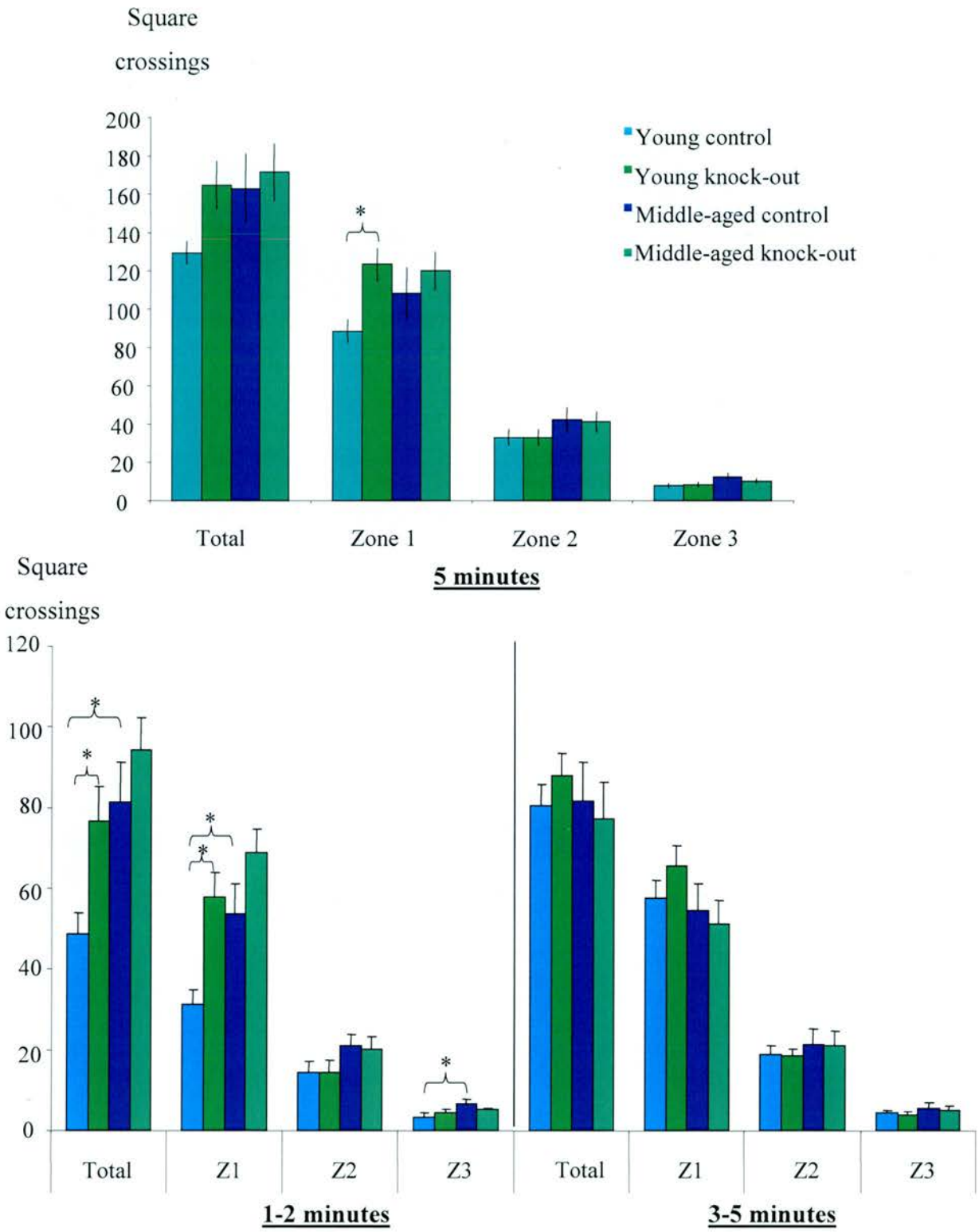

Figure 3-13: Square crossing in zones of the open-field-maze

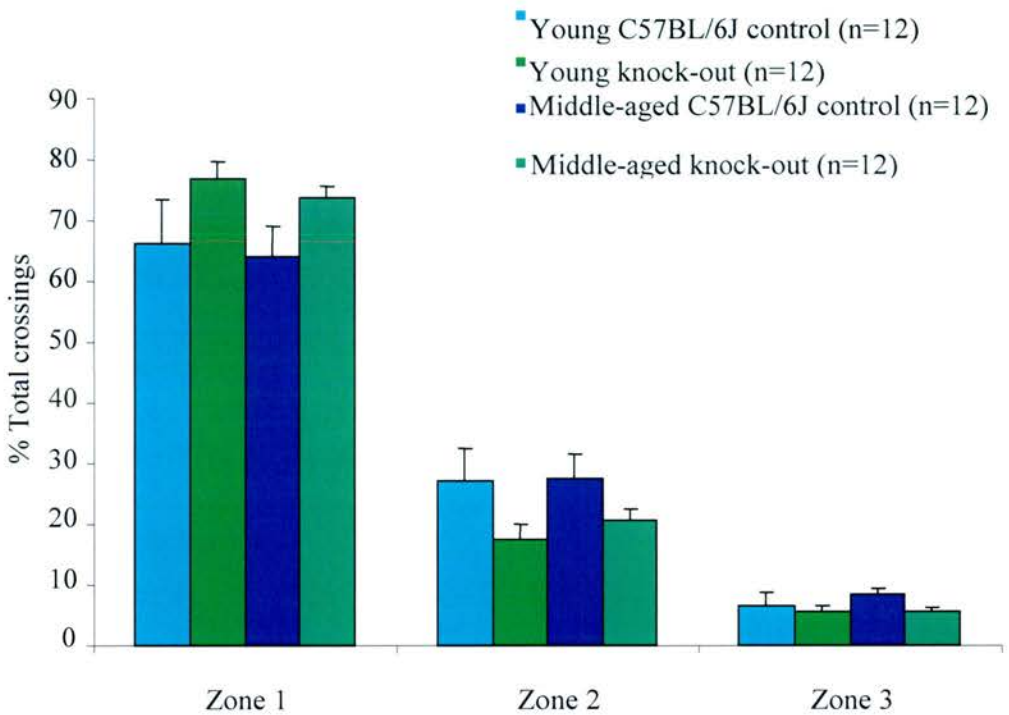

Figure 3-14: Proportional activity in zones of the open-field over the 5 minutes exploration

Proportional activity was calculated as the number of square crossings in a zone divided by the total number of crossings per animal. This measure would look at zonal preferences whilst controlling for changes in overall motor activity. ANOVA $p<0.05$ 11β-HSD1 knock-out *vs* control. There were no LSD post-hoc differences.

Table 3-6: Time spent non-motile in the open-field over the five minutes of exploration

*= $p<0.05$ LSD post-hoc analysis, young controls vs. young 11β-HSD1 knock-outs.

Group	Non-Motile (secs)
Young C57BL/6J control	100.3 ± 5.8 *
Young knock-out	49.7 ± 7.5 *
Middle-aged C57BL/6J control	80.2 ± 12.9
Middle-aged knock-out	70.2 ± 14.7

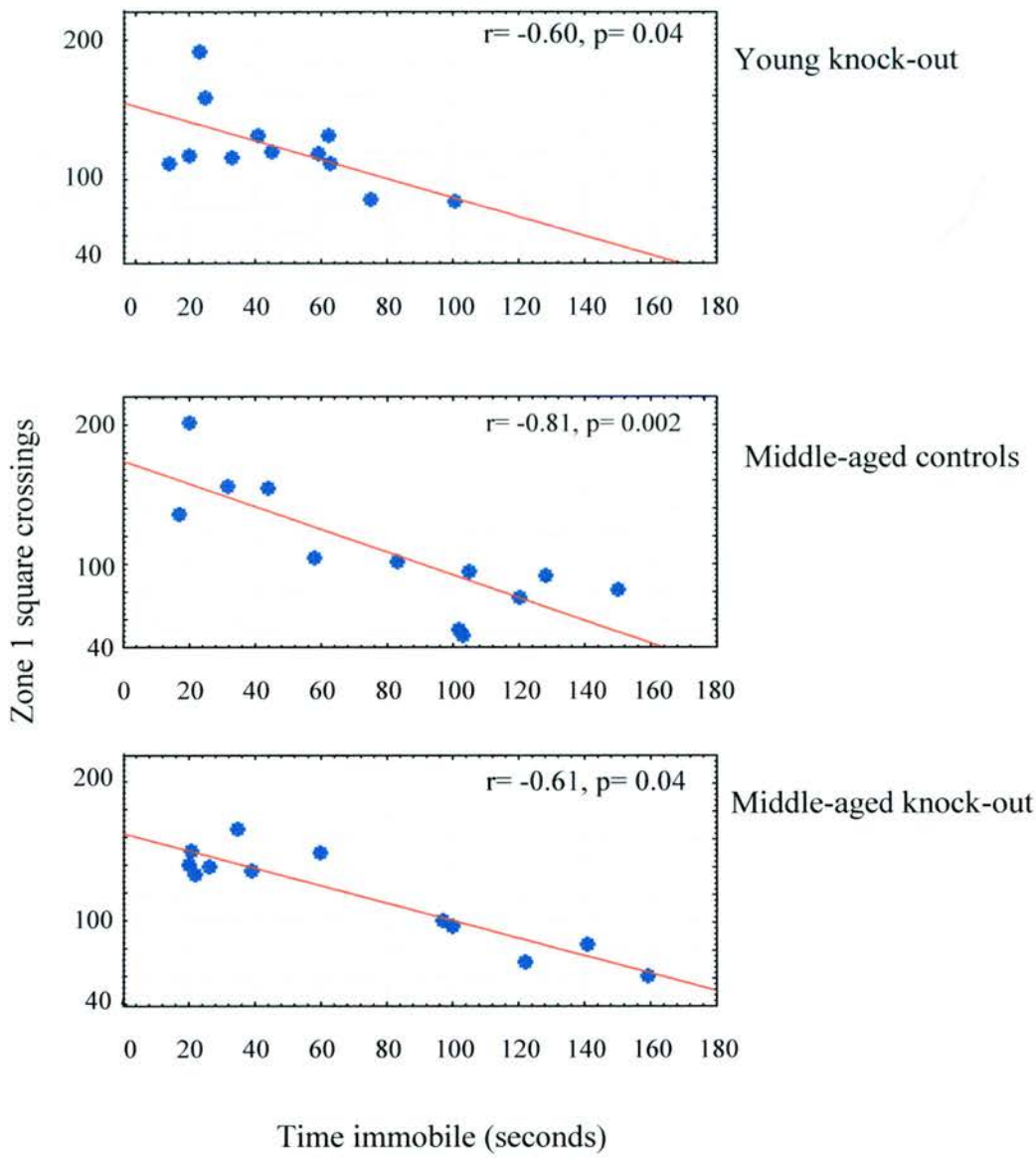

Figure 3-15: Correlations of immobility with activity in the outer zone of the open-field

There is a negative correlation between activity (square crossings) in the outer zone (zone 1) and time spent immobile in the middle-aged controls, middle-aged 11β-HSD1 knock-out and young 11β-HSD1 knock-out. This suggests that immobility accounted for individual differences in the numbers of squares crossed in the 5 minutes. There was no relationship in the young controls, suggesting that speed may have contributed more. The same relationships were true with total activity (data not shown).

Ethological measures – In order to further refine analysis of exploration measures of the number of vertical explorations and rears were made (Figure 3-16). There were no differences between groups over the 5 minutes.

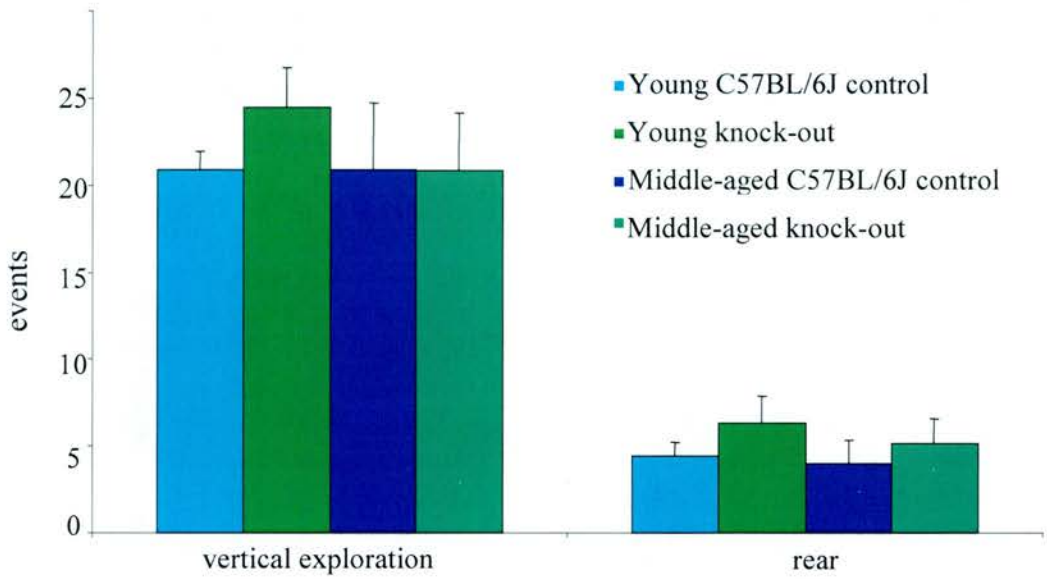

Figure 3-16: Ethological measures of exploration in the open-field-maze

There were no differences between groups in the performance of vertical exploration (against the side wall) or rears.

The Rota-rod

Errors - On the first day of training, there were significant differences in the abandoned attempts to stay on the rod at 4rpm for the initial 10 seconds ('errors') (Figure 3-17). There was a significant effect of age (aged animals making more errors than young) in the number of errors made on the first ($F_{(1,41)}$=12.47 , $p<0.001$)and second days ($F_{(1,40)}$=9.19 , $p<0.005$). The effect of the 11β-HSD1 gene was significant on the first day ($F_{(1,40)}$=3.92 , $p<0.05$). The number of errors decreased on day 2 and was close to zero thereafter. Post-hoc analysis results are shown.

Perseveration of running - All groups improved their performance from that of day 1 (Figure 3-18), but there was a difference in the rate of learning and level of performance. Both aged groups showed an impaired learning-curve (compared with young control C57BL/6J) and the young knock-outs reached a plateau earlier than the young controls. There were no significant differences on the first day of testing. Thereafter there was a significant negative effect of age upon time spent running (Day 2($F_{(1,40)}$=7.08 , $p<0.05$); Day 3 ($F_{(1,40)}$=5.98 , $p<0.02$); Day 4 ($F_{(1,40)}$=6.86 , $p<0.01$); Day 5 ($F_{(1,40)}$=19.28 , $p<0.001$)). On Days 4 and 5 there was a significant interaction of age and genotype (Day 4 ($F_{(1,40)}$=4.48 , $p<0.05$); Day 5 ($F_{(1,40)}$=4.00 , $p=0.05$)). Post-hoc analysis results are shown.

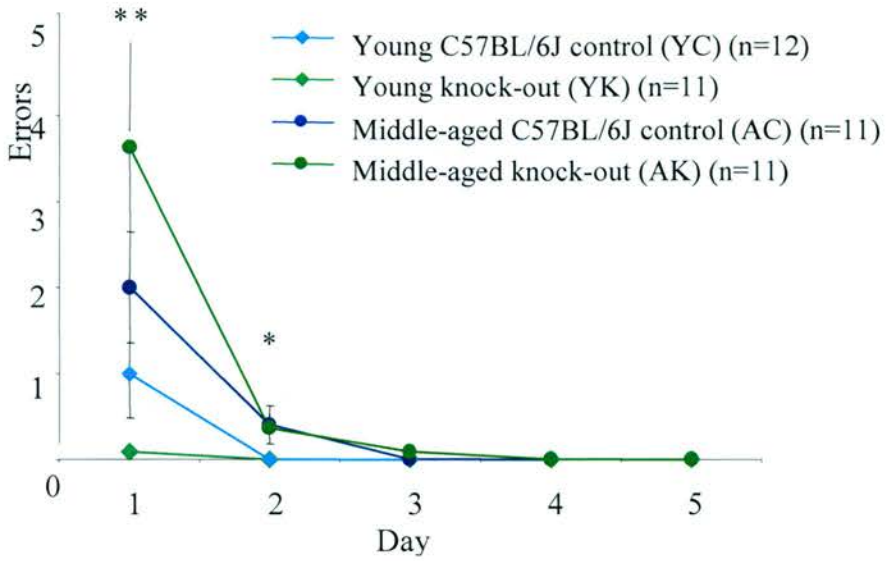

Figure 3-17: Number of errors made during the non-accelerating Rota-rod training

For the first 10 seconds of each trial, the rod was maintained at 4rpm. If an animal fell within this time, it was replaced for a maximum of 4 times (5 attempts). The middle-aged knock-outs made more errors than the middle-aged controls on days 1 and 2. By day 3, all animals could perform the task. Each*= $p<0.05$, **= $p<0.01$ LSD post-hoc analysis for middle-aged controls *vs* middle-aged knock-outs.

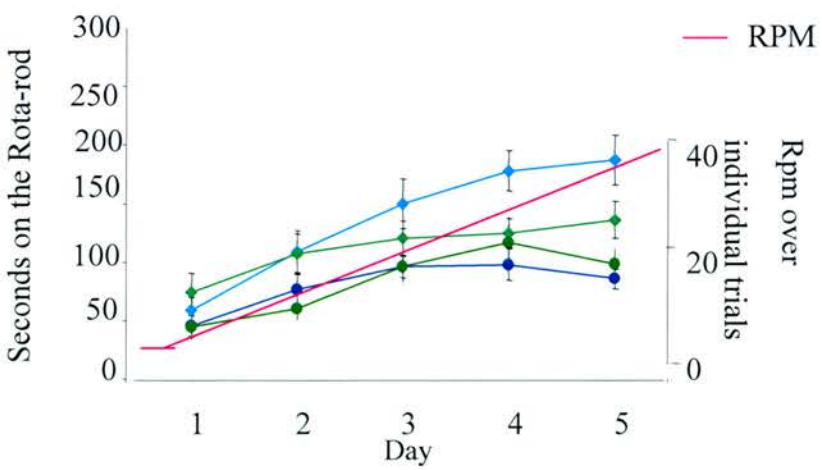

Figure 3-18: Time achieved on the accelerating Rota-rod

The chart shows the time spent on the rod (1 trial per day) against the speed of the rod achieved. It can be seen (RPM) that the rod began at 4rpm and the mice nearly achieved 40rpm by day 5. There were no post-hoc differences on the first day of testing. On Day 3, YC *vs* AC $p<0.05$. On Day 4, YC *vs* AC $p<0.005$, YC *vs* YK $p<0.05$. On Day 5, YC *vs* AC $p<0.0001$, YC *vs* YK $p<0.05$.

3.3.2. Plasma corticosterone

There was a significant increase of basal plasma corticosterone with a lack of 11β-HSD1 ($F_{(1,34)}$=10.16, p<0.005) (Figure 3-19). There was however no effect of age ($F_{(1/34)}$=0.75, p=0.39). The increase of plasma corticosterone was continued in the stress response to 5 minutes exploration of the Y-maze. There was an increase with a lack of 11β-HSD1 ($F_{(1,40)}$=23.44, p<0.00002) and with increasing age ($F_{(1,40)}$=13.03, p<0.001).

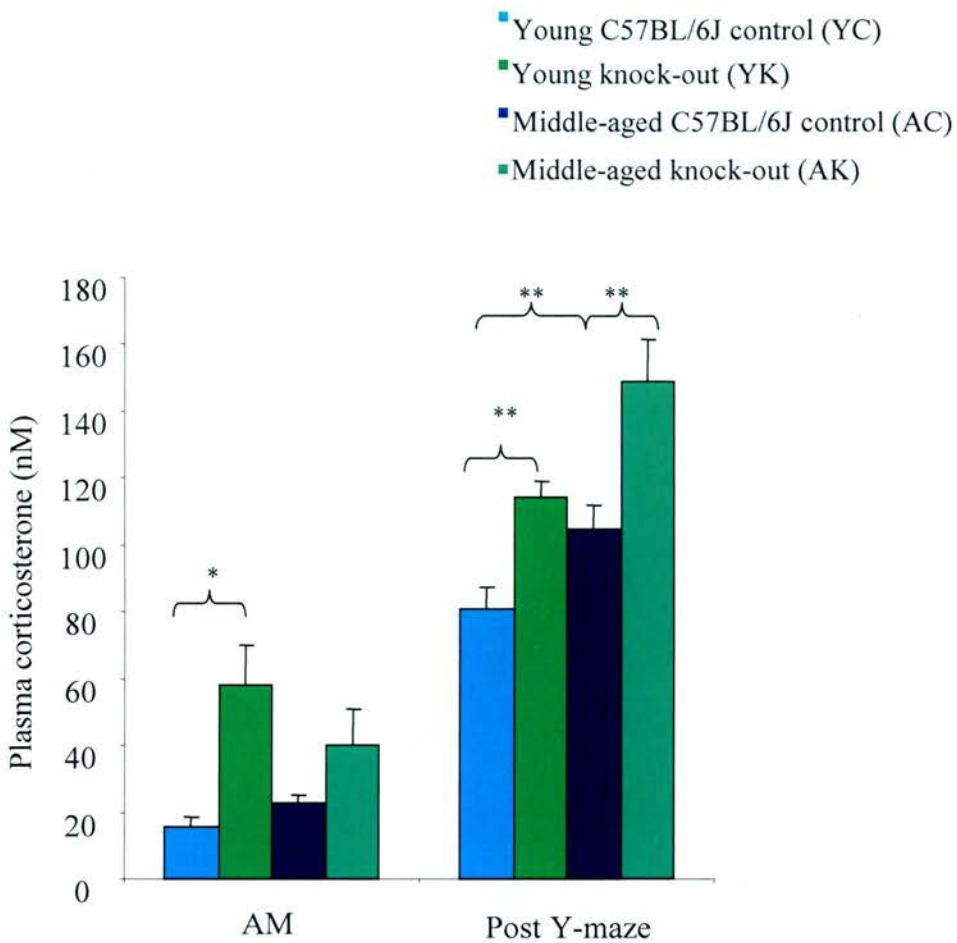

Figure 3-19: Plasma corticosterone values obtained at a control, diurnal nadir (AM) and after the 'spontaneous alternation exploration' of the Y-maze

Plasma corticosterone was analysed by radio-immuno assay from samples obtained by tail nick. Post Y-maze samples were taken directly after the spontaneous alternation exploration to assess the activity of the HPA axis during such exploration (YC (n=10), YK (n=8), AC (n=9), AK (n=11)). Control samples were taken precisely the same time of day (some weeks later) in the absence of a behavioural task (YC (n=12), YK (n=11), AC (n=9), AK (n=10)). *= $p<0.05$ and ** < p.005 LSD post-hoc analysis.

3.4. Discussion

The results from each behavioural test will be discussed, followed by the results from the analysis of plasma corticosterone and the relationship between the behaviours and glucocorticoids.

3.4.1. Behaviour

The Y-maze

The results from the spontaneous alternation test indicated that the young C57BL/6J control mice were able to navigate in an allocentric manner (rather than egocentric navigation which would have resulted in a low score). The potential random level of alternation was calculated to be 22% (based upon all 3-arm combinations).

After the 1 minute inter-trial-interval (ITI), most mice achieved a 40% exploration time in the 'novel' arm criterion; spending significantly more time in that arm than the 'start' arm and with no significant group differences in time spent in the 'novel' arm, nor in the proportions of mice achieving criterion. This indicated that all groups displayed a positive drive to explore novel spaces and that they were able form a spatial memory which was preserved for at least 1 minute. Equally, there were no overt short-term memory differences between the groups. The levels achieved were in line with other reports (Conrad and McEwen 1997; Dore et al. 2001). As other cues such as odours, large visual cues (global cues) and sounds were controlled, the assumption was that the visual cues were being used by the mice to navigate. In addition, when the number of visits to arms was analysed it was found that the

middle-aged control mice were less active than the young controls, which accounted for differences in activity in specific arms. The first choice of arm (after first exiting the 'start' arm) has been interpreted as a non-hippocampal function (Eichenbaum et al. 1994), thus its equivalence between groups in the current study does not contra-indicate hippocampal damage . After the 1 minute ITI, there were no differences in this parameter, the levels being similar to those of a previous report (Sarnyai et al. 2000), and there were no differences in the time taken to leave the 'start' arm.

After the 2 hour ITI, there were a number of changes in the exploration parameters discussed above. The middle-aged control mice were not significantly impaired when compared with the young control mice. However, a comparison of the percentage 'novel' arm exploration time showed that the performance after the longer ITI was signifantly impaired in the both young and middle-aged control groups, compared with the recognition trial after the 1 minute ITI. It was not impaired in the 11β-HSD1 knock-out groups relative to the 'novel' arm exploration after the 1 minute ITI. This result suggested that some aspect of long-term memory was improved in the 11β-HSD1 knock-out groups compared with the control groups. The 'novel' arm exploration (after the 2 hour ITI) was significantly reduced in the middle-aged controls compared with the middle-aged 11β-HSD1 knock-outs and a greater proportion did not achieve the 40% criterion. Together, these results indicated that the long-term spatial memory of the middle-aged knock-out mice was improved upon that of the middle-aged control mice, despite no revelation of age-related impairement in the control mice. When the number of visits to arms were corrected for overall activity (in which there were no differences), there were no

group differences in visits to the 3 arms of the maze. There were no significant differences in the proportions of mice choosing the 'novel' arm when they first left the 'start' arm. Despite, the middle-aged knock-out mice leaving the 'start' arm faster than the young knock-out group, there was no evidence of a speed *versus* accuracy trade-off.

The results from the Y maze did not show a clear age-related spatial learning impairment in the control groups. Our previous study had shown aged 129/Ola mice to be impaired in a watermaze task (Yau et al. 2001), which like the Y-maze was hippocampal dependent. In the current study, the older mice had only reached middle-age (12 months). It was possible that this was not old enough to reveal age-related deficits using this paradigm. Another study had shown a lack of spatial learning deficit, using the watermaze in aged mice of the same strain (Calhoun et al. 1998). However, the learning curve described there was steeper than expected for mice and the task used, may have been too simple to discriminate. A number of studies have indicated that cogninitive decline is more rapid in a subset of the population (Issa et al. 1990; Levy et al. 1994; Yau et al. 1995; Luparini et al. 2000; Sykova et al. 2002). If the current group of middle-aged C57BL/6J did contain such a subset, then it was possible that their decline had not reached a point where it would affect the group mean compared with the young controls. It was also likely that the 2 hour ITI was too stringent a test and that the study should have integrated an intermediate test (e.g. 1 hour). After the 2 hour ITI, the time spent in the 'novel' arm by the young controls was impaired compared with their time after the 1 minute ITI. This made the dissection of a negative effect in the middle-aged control mice

less likely. Despite this, there was evidence (time in the 'novel' arm, group proportion reaching criterion and equivalent performance after the 1 minute ITI and 2 hour ITI) that the spatial-memory of the middle-aged 11β-HSD1 knock-outs was improved compared with the middle-aged controls. There was some evidence that memory of the young 11β-HSD1 knock-outs was improved upon the young controls (equivalent performance after the 1 minute ITI and 2 hour ITI). It was possible that some factor played a role in the improvement of the middle-aged knock-outs in addition or instead of protection from age-related deficits.

The elevated-plus-maze

The low number of open-arm entries suggested that the maze was anxiogenic for these mice. A lack of differences in the number of open-arm entries may, therefore, be a consequence of this (such low open area entries have been published previously when investigating glucocorticoids and anxiety (Tronche et al. 1999)).

Arm exploration - There were no significant effects of age (C57BL/6J controls) upon the number of entries made into closed / open arms nor the time spent in those arms (refer to Figure 3-10). There was a small but significant increase in visits to the open arm made by the young 11β-HSD1 knock-out (compared with the young controls) in the first 2 minutes of exploration. Indeed, in the first 2 minutes, the young 11β-HSD1 knock-outs made a higher number of entries onto the open arms compared with the young controls. This may have been interpreted as a decrease in anxiety, but represented such a small number of visits that the significance should be regarded with caution. Despite the lack of an age-related change in visits to arms in the controls, the middle-aged 11β-HSD1 knock-outs made more visits than the

middle-aged controls to the closed arms over the 5 minutes. This was significant in the latter 3 minutes of exploration. 'Visits to the closed arms' has been used as an indicator of motor activity in the elevated-plus-maze and is not an indicator of anxiety (Carola et al. 2002). The finding that activity was increased in the knock-outs (by ANOVA) in the last 3 minutes, may be interpreted as a generalised increase in activity; related to loss of 11β-HSD1 and which increases during exploration of the maze.

Over the 5 minutes, there was a decrease in the time spent in the central region of the maze by both middle-aged groups (compared with their respective younger groups), which might be interpreted as a decrease in decision time (Carola et al. 2002). This difference was focused upon the latter 3 minutes, when it largely accounted for an increase in time spent in the closed arm by the middle-aged 11β-HSD1 knock-outs compared with the young 11β-HSD1 knock-outs.

Ethological measures - There were no age-related changes in open-protected stretches (OPS) over the full 5 minutes of exploration (refer to Figure 3-11) However, partial analysis showed a significant increase in OPS in the middle-aged controls compared with the young controls during the first 2 minutes of exploration. This relationship was reversed in the latter 3 minutes of exploration. The same pattern was seen in the knock-out groups, such that the older animals appeared to concentrate their risk assessment in the early part of exploration and the younger animals showed a delay. OPS is increasingly used as a correlate of decreased anxiety, even when no differences are found in closed and open arm measures of exploration (Rodgers et al. 1992). This could suggest differences in anxiety in young

and middle-aged animals at different stages of exploration or could be related to other factors associated with ageing. There was an increase in OPS over the full 5 minutes of exploration (concentrated in the latter 3 minutes), seen in the young 11β-HSD1 knock-outs compared with young controls. This result suggests that the young knock-outs were more anxious than their young controls, making more 'risk assessments'.

There were no differences between groups in the number of head dips made over the 5 minutes. However, the partial analyses showed a pattern similar to that of the OPS. There was an increase in head dips in both aged groups during the first 2 minutes (compared with relevant young groups) and a decrease in head-dips in the middle-aged 11β-HSD1 knock-outs compared with young transgenics in the latter 3 minutes.

A role for corticosterone in risk assessment was supported by a study in mice and rats which showed a positive correlation of corticosterone with stretched-attend, but not with open arm visits (Rodgers et al. 1999). It may be that intracellular corticosterone (due to 11β-HSD1 activity) is the mediator. There maybe influential differences between the groups, other than corticosterone. It may also be that the differences between groups are reflective of GR activation over an effect of MR activation. Future analysis would require frequent blood or intra-hippocampal sampling of corticosterone (perhaps by microdialysis) to clarify.

The lack of a side-wall on the open-arms and/or the colour contrast between the floor and maze may have heightened the anxiogenicity of this maze. Care should be taken interpreting elevated-plus-maze results from other mouse strains or the rat. In a study comparing the C57BL/6 with strain of 129/Ola, it was judged that the C57

performed most of its exploration in the closed arms (Montkowski et al. 1997). This may have contributed to the low number of open arm entries but in the quoted report the entries were higher than seen here.

The open-field

Analysis of the crossings in the different zones (adjusting for the different probability of entry in zones, based upon the different numbers of crossing points available for each zone) suggested that the mice were less likely to enter zones 2 and 3 (progressively) and that the inner zones were more anxiogenic (Carola et al. 2002).

An increased activity of the young 11β-HSD1 knock-outs compared with the young controls was shown in increased crossings (focused in the outer zone) and a decreased time spent immobile. In the same manner as used for further analysis of the Y-maze and elevated-plus-maze data, the number of square crossings in each zone was controlled for overall activity. It may be surmised, from the partial analyses, that this difference largely occured in the first few minutes of exploration. This analysis suggests that an effect of a lack of 11β-HSD1 in the open field is a subtle increase in 'anxiety', but it could also be interpreted as an increase in behavioural activation.

Increased exploration of the centre of the maze, without increased overall activity, has been used to identify anxiety behaviours and to effectively test the anxiolytic properties of a range of bezodiazepines (acting through $GABA_A$ receptors) and 5-HT inhibitors (particularly 5-HT_{1A} receptor antagonists and 5-HT re-uptake inhibitors) (reviewed in Belzung and Griebel 2001; Prut and Belzung 2003). Rats with ventral

hippocampal lesions showed no change in open-field behaviour (anxiety or locomotor) (Bannerman et al. 2003), however under mild stress conditions an increase in locomotion was seen. When the whole and dorsal hippocampus is lesioned in rats,then an increase in locomotion was seen (Bannerman et al. 1999). However, no change in locomotion was seen in whole hippocampus lesioned mice (Deacon et al. 2002). This result would suggest that the increase in locomotion seen in the current experiment was not simply due to increased hippocampal activity.

The time spent immobile was used as an inverse measure of the time spent mobile and could be used to estimate the speed of activity (squares crossed in unit time). In all but the young control group there was a negative relationship between time spent immobile and squares crossed in the outer zone. This suggested a positive relationship between time spent mobile and square crossings. In the young control group, it appeared that speed rather immobility / mobility accounted more for individual differences in the number of squares crossed. This could be further clarified by the use of an automated tracking system. There were no differences in the vertical exploration or rears made by the groups. A decrease in rearing was previously seen in mice with hippocampal lesions (Deacon et al. 2002).

The Rota-rod

All the groups appeared to learn the task of running the rod and of adapting behaviour to the acceleration of the rod. Performance of the C57BL/6J control was in line with other studies (Zeyda et al. 2001; Giese et al. 2001), considering procedural differences. The data revealed an age-related impairment in the middle-aged mice in terms of learning the initial procedure and of learning to adapt

behaviour. It has previously been shown that mice of this age are impaired in the constant speed Rota-rod (Thouvarecq et al. 2001) and this was supported by the 'error' data. Zeyda et al (Zeyda et al. 2001) suggested that performance in the acceleration task was not related to motor-ability per-se and so care should be taken in extrapolating to other tests. The ageing groups did not differ from one another. This was in contrast to the effect of the 11β-HSD1 absence in the performance of the aged animals in the spatial learning task and does not support a role for glucocorticoids (within 11β-HSD1 expressing cells) in ageing of the cerebellum. There is no evidence in the literature (to this author's knowledge) of glucocorticoid – associated ageing of the cerebellum.

However, the test did reveal that the young 11β-HSD1 knock-outs were impaired (compared with young controls) in their ability to learn the accelerating rod task.

3.4.2. Corticosterone

The knock-out mice showed higher levels of morning control and post-Y-maze plasma corticosterone. It should be noted that the basal levels suggest a slight stress response (presumably to handling or blood sampling). This could also be a product of the time of testing. Accurate nadir samples would be taken at approximately 08:00hrs. The base samples in this study were taken between 09:00hrs and 12:00hrs to correlate with the time of Y-maze testing.

An increase in plasma corticosterone at the diurnal nadir (08.00 am) and after stress has been reported previously in 11β-HSD1 knock-out (Harris et al. 2001; Yau et al.

2001). The study by Harris *et al* had shown decreased GR mRNA in the PVN. It had been inferred that the loss of enzyme was more influential upon the examined feedback than the change in GR mRNA because changes had been greater than had been found with a GR antisense (Karanth et al. 1997). An alternative (although not exclusive) explanation would be an increase in corticosterone production secondary to adrenal hypertrophy. Such increased production would have to over-ride feedback.

It can be hypothesised that all cells in the brains of the 11β-HSD1 knock-outs (excluding clearance effects) are exposed to equal levels of corticosterone. However, in the brains of the C57BL/6J controls, those cells which express active 11β-HSD1 are exposed to higher corticosterone. If the plasma corticosterone levels of the knock-outs were the same as those of the C57BL/6J controls, then expressing cells in the controls would be exposed to more corticosterone than correlating cells (without 11β-HSD1) in the knock-out. However, plasma corticosterone in the knock-out appears to be increased at the morning nadir and after stress. Therefore, the levels of corticosterone in expressing cells of the controls compared with correlating cells may have more, equivalent or less corticosterone depending upon the 11β-HSD1 reductase activity in each cell and the precise levels of plasma corticosterone. However, non-expressing cells in the C57BL/6J controls would be exposed to less corticosterone than correlating cells in the knock-out with higher plasma corticosterone.

Spatial learning and glucocorticoids

There have been several studies looking at corticosterone effects upon spatial learning. A body of work in the laboratory of McEwan has looked at performance in the Morris watermaze and in the Y-maze (Conrad and McEwen 1997; Conrad et al. 1999). The use of GR and MR agonists and antagonists has suggested that occupancy of MR is essential for spatial learning and that GR influences learning in a dose-dependent inverted-U manner (Conrad et al. 1999) in rats. The study showed differences in the time spent in arms rather than the number of visits, in a similar manner to the current study. There were no correlations with plasma corticosterone values found in the long ITI response-to-novelty test.

Interestingly, a mutant mouse with impaired GR dimerisation and DNA binding displayed impaired spatial watermaze performance (Oitzl et al. 2001). This was not improved by corticosterone replacement or fixing. Exposure of the current, experimental animals to the Y-maze resulted in a mild activation of the HPA axis. It is likely that this response would have increased over the next 30 minutes (Oitzl et al. 2001). Previous examination of HPA axis function in 11β-HSD1 knock-out mice (MF1) suggested that plasma corticosterone in knock-out mice would be elevated after a stressor (perhaps after Y-maze exploration) and that feedback upon that elevation would be delayed (Harris et al. 2001). The corticosterone results in the current study indicated that this phenotype would be seen in the current knock-out model (when tested). It could be anticipated that cells in the knock-out were exposed to increased peak levels of circulating corticosterone in the 2 hour inter-trial-interval, and to a longer exposure.

If the hippocampal distribution and levels of 11β-HSD1, GR and MR mRNA were similar in the brains of these C57BL/6J to those results described in the previous chapter, then it is valuable to draw together information from these two experiments. In the previous chapter there was a significant reduction of GR mRNA in the CA1 region of the hippocampus in the young 11β-HSD1 knock-out mice. However, levels of 11β-HSD1 mRNA were generally very low in most pyramidal neurons in this region. In the current study, there appeared to be a subtle protection from spatial memory deficit in the maiddle-aged mice with a lack of 11β-HSD1. Higher levels of corticosterone exposure over a lifetime could lead to impaired spatial learning, but in the present knock-out model, lower exposure of the cell to corticosterone in non-stressful conditions could help to prevent age-related impairments. This laboratory has previously shown lower tissue concentrations of corticosterone in the hippocampi of aged 129/Ola Knock-out mice, but this was in unstressed animals (Yau et al. 2001).

In the current study, the young control group did not appear to consolidate their spatial memory after the 2 hour ITI, the young knock-outs, however, did. Based upon work in the McEwen group (Conrad and McEwen 1997; McEwen and Lupien 1999), the results suggest a GR-mediated process. This could be due to higher or lower receptor activation. The fact that long-term memory (rather than short-term) was modulated, does indicate a DNA-mediated mechanism.

There have been other reports of manipulations which have caused effects in anxiety and in learning. A study involving a model for GR dysfunction has already been

referred to. The CRH-R1 knock-out has demonstrated an opposite phenotype, with decreased anxiety and cognitive ability (Strohle et al. 1998).

Future research – Due to time limitations in the current study, the role of 11β-HSD1 absence in protection from age-related spatial learning was only tested at middle-age. However, the mice were retested at old age (by other members of the laboratory) with spatial learning results which confirmed those found here (unpublished data). The use of a Y-maze in the current study, was useful compared with a classic watermaze, because it precluded the contamination of exploration by thigmotaxic effects. A version of the watermaze has been developed to perform the same control and to allow measurement of working memory and long-term memory (Diamond et al. 1999). It would be useful to examine the young and aged mice in such a version of the watermaze to further dissect aspects of learning. It may be useful to fix plasma corticosterone in knock-out animals (acute and chronic) to control for the high levels seen (Harris et al. 2001; Yau et al. 2001).

Anxiety, exploration and glucocorticoids

The results from these tests indicate a mild anxiogenic effect of loss of 11β-HSD1 in the young mice. This effect was lost in middle-age in the elevated-plus-maze and reduced in the open-field. The mild anxiogenic effect in the young (controls vs. knock-out) in the elevated-plus-maze was seen as the increase in OPS in the latter time bin, this being when the young increased their risk assessment behaviour. Conversely, differences were seen in the Open-field in the first 2 minutes. It appeared that the elevated-plus-maze was a more stressful exposure than the open-field, an observation made previously (Montkowski et al. 1997) (an alternative

explanation is that the mice were more habituated to handling during the Open-field because of the order of experimentation).

Most floor square-crossing activity occurred in the first few minutes when corticosterone would have been closer to basal; the mice were well handled and plasma corticosterone values should have been low. Correlations of square crossing activity and time spent immobile suggested that speed accounted for individual differences in activity in the young control mice, but that stopping contributed significantly to individual differences in the other groups. This 'stopping' may have been inactivity, vertical exploration (there were no group differences) or 'freezing'. 'Freezing' behaviour (an anxiety related response to a threat) has been proposed as an MR-mediated behaviour (reviewed in Korte 2001), which is dependent upon GR developmentally. However, activation of hippocampal MR has been correlated with increased activity in a light-dark box task (Smythe et al 1997). In light of a new understanding of MR occupancy at low / intermediate levels of corticosterone, the elevated levels of plasma corticosterone in the current study may have lead to increase MR activation. GR should become progressively more involved. In a model of impaired GR function, the transgenic mice demonstrated no changes in motor behaviour, but they did spend less time in the centre of an open-field (Strohle et al. 1998) (notably, visiting the open arms of an elevated-plus-maze more).

It is difficult, from these results, to interpret these differences as a change in State or Trait anxiety. An adrenal response may have been experienced within the 5 minutes of exploration, leading to rising levels of plasma corticosterone. With a basal corticosterone level, neurons lacking the enzyme would be exposed to less

intracellular corticosterone. The area tightly associated with anxiety is the amygdala. This author found no evidence of high expression of 11β-HSD1 in the amygdala (observation only in 129/Ola mouse and rat brain). Low levels of 11β-HSD1 in the amygdala have been previously reported, although the nucleus was not identified (Moisan et al 1990). It is possible that cells of the amygdala were exposed to higher corticosterone in the young 11β-HSD1 knock-outs compared with the young controls (refer to discussion of corticosterone values) and that this was related to the anxiety or exploratory behaviour seen. Further exploration of 11β-HSD1 in the amygdala may help to shed light upon the anxiety-related behaviours shown here. Interestingly, a CRH over-producing mouse model showed reduced activity in the open-field (van Gaalen et al. 2002), with no zonal differences. CRH has not been explored in the hypothalamic sites of the 11β-HSD1 knock-out brain. In the amygdala, CRH is positively regulated by corticosterone

Future research - This study of anxiety and exploration was preliminary. In order to further explore a phenotype associated with the loss of 11β-HSD1 it would be useful to use a range of controls. To control for the confusion about local corticosterone levels, it would be useful to include groups of mice with fixed plasma corticosterone. This could be achieved through adrenalectomy and corticosterone replacement. It would also be useful to include some controls of anxiety behaviour. This could be achieved through the use of an anxiolytic agent. Care should be taken in the choice of agent, because they can show differential effects upon exploratory behaviours in the elevated-plus-maze. Low dose benzodiazepines can reduce open arm avoidance and risk assessment but $5HT_{1A}$ anxiolytics only decrease risk

assessment (reviewed in Belzung and Griebel 2001). The tests used in this thesis should be combined with other tests of anxiety and exploration. This would allow for factoral analysis, which may improve understanding of the precise behavioural phenotype. The use of the hole-board test could allow examination of exploration, dissociating the confounding parameter of changes in activity (Deacon et al. 2002; van Gaalen et al. 2002).

Motor learning and glucocorticoids

There is not a wealth of work looking at the effects of corticosterone upon motor learning. However, a similar acute effect to that muted for the spatial learning is possible. It was possible that local levels of corticosterone in the middle-aged mice, influenced the ability to learn the rota-rod task (in the non-accelerating verson phase). The middle-aged 11β-HSD1 knock-out mice making more errors than the middle-aged controls. There is no published direct, evidence of glucocorticoid-related ageing impairments of cerebellar activity. It is likely that the differences seen between the young control and young 11β-HSD1 knock-outs in the accelerating phase of the task are related to differences in their plasma corticosterone. However, the middle-aged groups did not differ in this phase of the task and thus a role for 11β-HSD1 could not be shown. These results do no dismiss a possible role for developmental influences of changes in local corticosterone in the developing brains of the 11β-HSD1 knock-out mice. A report by Zeyda et al looking at somatostatin null mice showed a similar impairment (Zeyda et al. 2001). It is interesting that these knock-out mice also exhibited increased morning corticosterone levels.

Future research - A number of further studies would be necessary to clearly answer whether / how a life-time absence of 11β-HSD1 affected spatial learning in the young and middle-aged mouse. An extensive HPA axis profiling of the knock-out on the C57BL/6J background would be essential, but at the time of this project was the subject of study by another member of the laboratory. It would also be useful to sample for plasma corticosterone at the time of each behavioural test.

3.5. Summary

The experiments in this chapter indicated that the phenotype of protection from age-related hippocampal-dependent spatial learning previously seen in aged 11β-HSD1 knock-out mice (129/Ola background) was likely to be replicated in the knock-out on a C57BL/6J background. The mice tested here were middle-aged and there were not clear age-related deficits seen; however, the middle-aged 11β-HSD1 knock-outs were showed significantly improved long-term memory (no difference in performance after a short or long-term inter-trial-interval) and were more likely to reach a performance criterion compared with the middle-aged control group. There was also some evidence of improved long-term memory in the young knock-outs compared with the young controls. Short-term memory and searching strategy were not affected in any of the groups. These results suggest a change in GR activation (either increased or decreased), which enhanced long-term memory (specifically) and some protection from the early stages of age-related cognitive decline. There was evidence of a mild increase in anxiety in the young 11β-HSD1 mice. It was not clear whether this was a response to exposure/ novelty (State) or a base (Trait) anxiety; and did not uphold the hypothesis that anxiety would be reduced. The results from the open-field could be interpreted as an increase in behavioural activation, which was not seen in the elevated-plus-maze. An ageing deficit was revealed on the motor-learning task, but the knock-outs were impaired compared with the controls. The significant effect of 11β-HSD1 upon motor-learning suggested an elevated corticosterone effect in the 11β-HSD1 knock-out.

The data points to a complex of cells exposed to comparatively high and low levels of glucocorticoid. It is essential to identify these cells if the phenotype is to be understood. In future exploration of the role 11β-HSD1 in behaviour and ageing, brain-specific 11β-HSD1 knock-outs would be invaluable. This would help to clarify specific effects of 11β-HSD1 and ageing upon exploration, behavioural activation, anxiety and ability to interpret to interpret danger cues. In addition, controlling plasma glucocorticoid levels would help to limit the variables involved.

Chapter 4

11β-HSD1 in Primary Cultures of Rat Brain Cells

4.1. Introduction

11β-HSD1 mRNA has been demonstrated in various areas of the rodent brain (Brereton et al. 2001; Diaz et al. 1998; Lakshmi et al. 1991; Moisan et al. 1990). It has previously been shown that primary hippocampal cultures display 11β-HSD1 reductase activity (Rajan et al. 1996) in intact cells. It would be of interest to explore activity in cells from other regions of the brain. Should these cultures display appropriate activity, they could act as models for adult cells.

The previous chapters have described a protection from glucocorticoid and ageing-associated memory impairment in a transgenic mouse which did not express 11β-HSD1. It was also found that there was a mild anxiogenic effect in these mice and a significant impairment in motor learning. In light of these results and the distribution of the enzyme in brain, it would be of interest to investigate enzyme activity in cells of the frontal cortex. In addition, considering its role in motor learning and water-maze performance, it would be essential to model activity in the cerebellum.

4.1.1. Considerations Made in the Use of Primary Cultures to Model Adult Cells

There are several considerations to be made before deciding to use primary culture as a model of enzyme activity in the adult. The expression of 11β-HSD1 and the dehydrogenase 11β-HSD2 has been shown to change in the developing brain in a complex manner (Brown et al. 1996; Diaz et al. 1998). It should be considered that some of the areas to be investigated in the current experiment express both isozymes throughout development (at different stages) and that this may affect gross steroid conversion in assays (refer below). It should also be considered that the process of culture may affect differentiation of the cells in different ways. More positive

arguments are that cultured cells could be used in the future to model molecular control of the 11β-HSD1 gene or to create tissue specific transgenes. In addition, when interpreting the results of the previous chapters, there have been problems isolating local from more global corticosteroid effects. Primary cultures could be used to focus upon local effects. This technique was used previously to look at the effects of antidepressants upon GR in hippocampal pyramidal neurons directly (Okugawa et al. 1999).

4.1.2. 11β-HSD1 *in vitro*

The activity of 11β-HSD1 *in vitro*

The activity and control of 11β-HSD1 has now been modelled in a variety of cell lines and primary cultures. As discussed previously (Chapter 1) there has been some confusion over the activity of 11β-HSD1. Does it act as an 11β-dehydrogenase like the type-2 isozyme or as a reductase? The use of cultured cells has now been used to establish that liver-derived cDNA encodes a reductase in COS-7 cells (Low et al. 1994a). It has been shown to act as a reductase in primary cultured, rat Leydig cells (Leckie et al. 1998), hepatocytes (Jamieson et al. 1995) and hippocampal pyramidal neurons (Rajan et al. 1996). Much of the confusion over reaction direction came from historical association with the type-2 isozyme and from results from homogenates, where the type-1 acts predominantly as a dehydrogenase (Rajan et al. 1996). In the current study, it was anticipated that the enzyme would act as a reductase in the adult cells, but this had to be substantiated. In addition, it had to be determined whether cultured cells would act as models for adult expression.

The control of 11β-HSD1 *in vitro*

Hormonal control - A number of studies have looked at the hormonal and pharmacological control of 11β-HSD1 *in vitro* and developmental control *in vivo.* 2S FAZA cells have been used to model hormone control in hepatocytes (Voice et al. 1996). Dexamethasone was shown to increase transcription, suggesting a GR mediated mechanism. This was in opposition to the control exerted by forskolin (a G protein / cAMP agonist) and insulin. However, they potentiated the increase by Dexamethasone. It has subsequently been shown that hepatic 11β-HSD1 is also under regulation by transcription factor C/EBP (CAAT/ enhancer-binding protein) family members (Williams et al. 2000). It is not known whether this control translates to the brain.

Pharmacological inhibitors - There have been several pharmacological inhibitors of the type-1 isozyme used in studies. Their value may be limited by a lack of specificity for either of the two isozymes. In a study looking at inhibition of the human type-1 isozyme reductase activity in transfected yeast; glycerrhitinic acid (a liquorice derivative), carbenoxolone (the hemisuccinate ester of the latter) and furosemide (a therapeutic diuretic) were potent inhibitors (Hult et al. 1998). In the same study, Dexamethasone was an intermediate inhibitor of cortisone 11β-oxo-reduction; although 11-dehydrocorticosterone has been shown to be a substrate in a later study (Diederich et al. 2000). Notably, for this thesis, glycyrrhetinic acid has been used to inhibit feedback of the HPA axis (Seckl et al. 1993), where it was likely acting through inhibition of 11β-HSD1.

4.1.3. The Ontogeny of the 11β-HSD Isozymes in the Brain

The enzyme transcript is also under complex control during development. There appears to be a balance between expression of the type 1 and 2 isozymes. The author's laboratory looked at the distribution of both isozyme transcripts in the developing rat brain (Diaz et al. 1998). In general there is widespread expression of the type-2 isozyme in early development, which becomes progressively more restricted into the third trimester. The type-1 isozyme, is present in the early extra-embryonic membrane, but then is almost absent until the third trimester. With reference to the areas of interest to the current project, the neo-cortex expressed the type-2 isozyme until mid-gestation, but there was no significant expression of the type-1 isozyme (the study finished at embryonic-day 22.5). The hippocampus can be seen to express the type-2 isozyme at embryonic-day 11.5, but thereafter expression of the type-1 can be seen from embryonic-day 17.5. The cerebellum could be seen expressing the type-2 until late-gestation (at least), but was shown expressing the type-1 at embryonic-day 19.5.

A potential role for glial 11β-HSD1 in the brain

The relatively high proportions of glia in the originally described hippocampal cultures (Rajan et al. 1996) suggested a possibility of activity in the glial population. In addition, many of the expressing cells which were seen in the rodent hippocampus remained unidentified (refer to Chapter 2), some glia had previously been found immuno-positive for the 11β-HSD1 (Brereton et al. 2001). There is a wealth of

literature regarding steroidal metabolism by glia (reviewed in Jordan 1999). In addition, glia should be of interest to anyone investigating the ageing brain. Steroid-related changes in glia have been described in the ageing brain (reviewed inNichols 1999) and *in vitro* study have shown them to be endangered (like neurons) by glucocorticoid exposure (Horner et al. 1990). Glia in the dentate gyrus have also been identified as the precursors of new granular neurons (Barres 1999) and are involved in the apoptosis of granular neurons after adrenalectomy (Bye and Nichols 1998).

4.1.4. Aims

The chapter describes the general culture and analysis methods used, then progresses to describe specific experiments and their results.

Models to be investigated

The hippocampus, cerebellum and frontal cortex express active 11β-HSD1 in the adult brain (Moisan et al. 1990; Lakshmi et al. 1991). Primary hippocampal cells have been used to model the consequences of activity in the adult ageing brain (Rajan et al. 1996). It would useful to future research into the role of 11β-HSD1 in the ageing brain, to comparatively examine the control and consequences of 11β-HSD1 activity in cells from the hippocampus, cerebellum and frontal cortex. Thus the suitability and activity of cultures of cerebellar granular and cortical pyramidal neurons were investigated, to examine the viability of such models for future research. In the current study, hippocampal cultures were used to establish

methodology and to act as a positive control. The primary culture of cerebellar neurons was new to the laboratory and was developed in the course of this study. The specificity of glucocorticoid conversion was briefly examined in cerebellar granular neurons by examination of pharmacological inhibition by known inhibitors of 11β-HSD1 activity.

Investigating the role of glia in 11β-conversion in vitro

In the current study, it was thought that the significance of glia in steroidal conversion could be initially explored by examining enzyme activity in cortical and hippocampal cultures of differing neuronal/glial proportions.

4.2. General Methods and Materials

4.2.1. Primary Hippocampal Pyramidal Neuron Culture

This method was after that performed by Dr M Lai in the author's laboratory (Lai et al. 2003).

Solutions

Recipes for the 'dissection media', the 'plating media' and the 'chemically defined culture media' can be found in Appendix C. Ingredients for the 'dissection media'and 'plating media' were sterile and prepared in the culture hood. Ingredients for the 'chemically defined culture media' were not sterile, but the solutions were prepared and double filtered (0.2μm) (Sartorius) within a culture hood. All solutions were warmed to 37°C before use.

Dissection

Gross dissection - Cells for primary hippocampal culture were taken from embryonic-day 18 rat embryos. Extracting the hippocampi at embryonic-day 18 ensured that the anatomical structure has developed but minimised the proportion of glia. Granular neurons, from the dentate gyrus, do not differentiate until after birth.

Cross contamination (by bacteria from the skin) was reduced by the use of sterile dissection equipment and the use of different equipment for each stage of dissection. Dissection was performed under a cold light and was performed quickly to prevent the death of neurons.

The pregnant female was sacrificed quickly by cervical dislocation, without damaging the abdomen. The abdomen was swabbed with alcohol and opened, without rupturing the uterus or intestine. A small, pointed pair of scissors was used to open the uterus at one end and the uterine sacs transferred individually to a Petri dish. Each embryo was then be removed from the sac and decapitated quickly. The heads were placed in a clean dish and stabilised by inserting fine forceps into the eye-sockets (dorsal surface uppermost) and fine scissors were used to dissect-out the brain. The scissors were inserted into the back of the skull where the spine exits and an incision made on the left or right side, across the top of the ear to a point on top of the skull. This was repeated on the other side until the two incisions met. Care was taken to prevent damage to the brain as the skull top was lifted off. Any obvious meninges were cut to prevent damage to the brain and the brain was teased from the skull. The brain could be gently removed from its anchor by touching it onto a small pool of 'dissection media' in a clean dish, the surface tension pulling the brain out.

Isolation of the hippocampi - Once all of the brains had been collected, the hippocampi were dissected out. Using a pair of fine, pointed forceps (for manipulation) and a pair a fine, curved forceps (for hippocampal removal) the brain cortices were opened. The pointed forceps were used to anchor the cerebellum/brain stem and the outside curve of the curved forceps was used to open each hemisphere (individually) such that the brain had the appearance of a 'butterfly'. The hippocampus could be seen as a pale arc along the middle of each 'wing'. The outer curve of the forceps was used to gently grasp the 'arc' and lift it from the brain. The hippocampus was then placed in a fresh, shallow pool of media (too much media

maked observation difficult). When all hippocampi had been removed, they were cleaned. Any obvious meninges (red in colour) were removed. It was essential to only collect structures which were obviously hippocampal and to avoid cortical contamination. The hippocampi could be seen as a translucent ribbon with a more opaque line running down the longer centre. Any other structures were removed. This reduced the yield but increased purity. The cleaned hippocampi were pooled appropriately in the 'dissection media' in a clean Corning tube and the cells cultured immediately.

Hippocampal culture

Cell dissociation - The tubes were transferred to a sterile culture-hood (preferably for dedicated primary culture use), the hippocampi transferred to a sterile 2ml eppendorf and washed three times with 1 ml 'dissection media' (refer to Appendix C). The 'dissection media' was removed and the cells incubated in trypsin (Gibco 25300-054) (final 1mg/ml) in sterile water, for 12 minutes at 37°C without agitation. The hippocampi were now delicate and clumped by DNA strands. The trypsin was removed (being careful to avoid the DNA strands and loss of cells) and the trypsinisation was stopped with the addition of 1ml of the 'plating media'. The tube was agitated with 2 taps of a finger and stood for 90 seconds. The media was then removed and the hippocampi washed three times with 1ml of 'dissection media'. 1ml of 'plating media' was then be added to the hippocampi, which were then triturated with a sterile glass pipette, approximately 15 times. Final dissociation was achieved by gently taking the cells through a 25 gauge needle (25G) (1ml syringe) three times (disposing of clumps) and dispensing the resulting cloudy media into

5mls of 'plating media' in a fresh 15ml Corning tube. This was then spun at 600rpm for 6 minutes, at room temperature. The tubes were removed carefully from the centrifuge, so as not to disturb the pellet. Glia could be seen against the inclined sides of the tube (determined by immuno-histochemistry during protocol development). The media was removed (for disposal) from the tube with a sterile glass pipette in a circular movement which removed the glia from the sides. 5 mls of fresh 'plating media' was then added to the tube and the pellet re-suspended by tapping the tube.

Plating-out - The cell density was estimated to facilitate consistent plating density. 20µl of cells were counted in 20µl Trypan blue (Sigma) on a 'Neubauer Improved Haemocytometer'. The number of 'bright' cells was counted and a $2x10^4$ adjustment applied to calculate the number of cells per ml (cultures with over 10% cell death were abandoned). Cells were diluted in 'plating media' to $1x10^6$ cells/ml and plated at 2mls in 6-well poly-L-lysine treated culture plates (Beckton and Dickson 356413) which had been brought up to room temperature.

The cells were then held in an incubator at 37°C / 5% CO_2 in the 'plating media' with 'foetal calf serum' for 24 hours to allow 'plating down'. The 'plating media' was removed and replaced with a warmed 'culture media' (without allowing the cells to dry). Previous analysis of the 'foetal calf serum' showed significant levels of corticosterone, hence further incubation was in 'chemically defined media'. The new media was supplemented with 1µl/ml Gentamicin, 2.5µl/ml glutamine, 5µM Arabinoside C (Sigma C6645)(AraC). The AraC inhibited the proliferation of glia at this concentration, such that neuron:glia ratios of 19:1 could be achieved. This ratio

was adjustable by modulating the glial proliferation inhibitor treatment or inhibiting neuronal adhesion to the culturing surface (see below). The cells were used on the seventh day of culture.

4.2.2. Primary Cortical Pyramidal Neuron Culture

The general method was based upon that used by Dr Macleod (Macleod et al. 2003) with some modifications.

Solutions

The dissection, plating and chemically defined media were as those used for the primary hippocampal culture. The dissagregation media (detailed in Appendix C) should be prepared fresh, double filtered (0.2μm) and warmed to 37°C in advance of the dissection.

Dissection of cortices

The general principles of aseptic technique were as discussed for hippocampal culture.

Brains were taken at embryonic-day 18 and removed from the skull as discussed for hippocampal culture. The frontal cortices were opened (as described) and the hippocampi removed to prevent cell contamination (affording the opportunity to perform a simultaneous hippocampal culture. The opened cortex was then removed from the midbrain into a clean petri dish with shallow 'dissection media'. The olfactory lobe was removed and the cortex placed with the meninges uppermost. If

gentle pressure was applied to the middle of the cortex (with curved forceps) the meninges could be caught and removed with pointed forceps. The cortex was then be chopped (approximately 0.2mm intervals) with single perpendicular passes.

Cortical culture

The chopped tissue was transferred to the 'trypsinisation solution' and incubated at 37°C for 12 minutes. The 'trypsin inhibitor' was then added and the tube was gently rocked to aid mixing, then centrifuged at 1000 rpm (200g) for 5 minutes at room temperature. The supernatant was discarded and the pellet re-suspended in the 'trituration solution'. The cells were then triturated with a glass pipette (12x) and 3 passes through a 25G needle; then spun at 1000 rpm for 10 minutes and the supernatant removed (refer to hippocampal culture method). The pellet was re-suspended in 10ml plating media. The viable cells are counted (refer to hippocampal cell culture) and the volume adjusted, such that cells were plated at 0.5×10^6 cells / ml.

The cells were incubated. After 1 day in culuture the media is wholly replaced with culture media. Cells were harvested / used on the seventh day of culture.

4.2.3. Cerebellar Granular Neuron Culture

The general method was based upon that described by Martin Cambray-Deakin (Cambray-Deakin 1995) with some modifications.

Solutions

Stocks of reagents were prepared in Earle's Balanced Salt Solution (refer to Appendix C), aliquoted and stored at -20°C. Solutions were prepared in a culture-hood, double filtered and warmed to 37°C before use (refer to Appendix C).

Dissection of cerebella

Granular neurons could be cultured from brains taken from days *post-partum* 6 to 8. The earlier the dissection the cleaner the culture, however, this is offset by a reduced yield. In the interests of consistency, brains were all taken at *post-partum* day 7. At this stage of development the Purkinje neurons would have developed but their differentiation would not allow survival (Tabata et al. 2000).

The general rules regarding contamination were similar to those for hippocampal culture. However, it was the author's opinion that the risk of contamination was increased because the brains were taken *post-partum* and the skin had an increased risk of contamination.

Brains could be dissected one culture at a time. Contamination was limited by using separate instruments at each stage of dissection. The head was removed by careful (but rapid) decapitation. Using a separate pair of smaller scissors and a blunt pair of forceps the snout was grasped and the skin removed from the top of the skull. The

skull was opened by making an incision at the start of the spinal column. The incision then progressed along one side, above the ear to the top of the skull. This was repeated on the other side. The top of the skull was lifted off, the meninges cut and a small spatula used to break the optic nerves and remove the brain. The brain was placed in a clean petri dish (dorsal side up) and the cerebella were removed by blunt dissection between the cerebellum and the colliculi. Retaining the pons aided manipulation, as the cerebella were transfered to a sterile, roughened acrylic tile. The pons was removed by blunt dissection and the meninges were removed with fine forceps. The roughened tile surface assisted in the removal of the meninges. The cerebella are then chopped with a flat-blade scalpel with two perpendicular, single-pass series of chops at approximately 0.2mm intervals. The tissue was then transferred to the 'dissection buffer' in a 50ml Corning tube.

Granular culture method

Cell dissociation - The tube was immediately transferred to a culture-hood and the buffer removed. The tissue was then washed three times with Earle's Balanced Salt Solution and the tissue was transferred to a fresh 15ml Corning tube with 'trypsinisation buffer' (refer to Appendix C). The tissue was incubated at 37°C for 15 minutes, agitating frequently. Most of the buffer was then removed and the 'trypsin inhibition buffer' (refer to Appendix C) added. The tube was inverted three times to dissolve the leached DNA and was centrifuged at 180g, 10 seconds at room temperature. The supernatant was then discarded and the cells gently resuspended in 1.5ml 'disaggregation buffer' (refer to Appendix C). The cells were then be triturated 12 times, allowed to settle for 2 minutes and the supernatant transferred to

a clean tube. The supernatant contained the disaggregated cells and the connective debris was retained in the pellet.

Plating-out – The supernatant was underlain with 2ml of the 'gradient buffer' (refer to Appendix C) with a sterile glass pipette. The tube was then carefully transferred to the centrifuge and spun for 5 minutes, 180g at room temperature. The supernatant was discarded (as described for the hippocampal culture) and the pellet was resuspended in 5 ml 'plating media' (refer to Appendix C). The cell density was established (as described previously) and the volume adjusted such that there was a cell density of $1x10^6$ cells per ml. The cells were then plated in warmed poly-L-lysine coated 6 well plates at 2ml per well.

The cells were incubated for 24 hours in the media, to allow 'plating down'. The media was then changed for the culture media with stripped 'foetal calf serum' (from which glucocorticoid had been removed) (refer to Appendix C). At this point 80μM Fluorodeoxyuridine (FDU) (Sigma F0503) (prepared in EBSS) was added to inhibit glial proliferation. The cells were harvested or used on the seventh day of culture.

4.2.5. Evaluation of Gross Dehydrogenase / Reductase Activity

Gross dehydrogenase / reductase activity of 11β-HSD1 was estimated by incubation of the culture with tritium-labelled steroid, either corticosterone (measuring dehydrogenase activity) or 11-dehydrocorticosterone (measuring reductase activity). Estimation of conversion was achieved by thin-layer-chromotography.

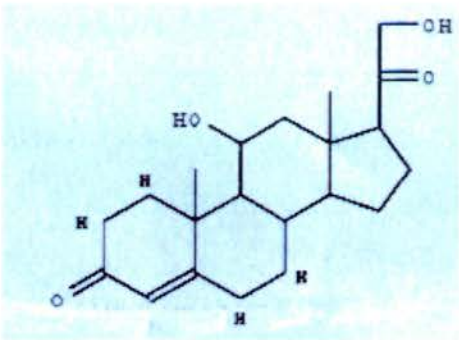

Figure 4-1: Tritiated corticosterone

Corticosterone (Amersham) was tritiated at the 4 hydrogens shown.

^{3}H-11-dehydrocorticosterone generation

Tritiated corticosterone could be purchased (Figure 4-1), but tritiated 11-dehydrocorticosterone had to be produced by incubation of tritiated corticosterone with 11β-HSD2. Rat placenta contains active 11β-HSD2 (obtained when removing embryonic-day 18 pups) was incubated with ^{3}H-corticosterone (refer to Appendix C). It was essential that all solvent (notably Toluene) was evaporated from the ^{3}H-corticosterone, any remaining would prevent conversion. The mixture (in a glass tube) was mixed and incubated for 3 hours at 37°C (protected from direct sunlight) in

a moving water-bath. The reaction mixture was then split into 5 glass tubes and the reaction stopped with 2ml ethyl acetate to each tube. After mixing well, the tubes were then centrifuged at 2000rpm (4°C) for 15 minutes. The upper, clear organic phase was transferred to a scintillation vial and dried down (under O2-free N2), then re-suspended in 250µl ethanol and stored at -20°C.

Purity, as assessed by TLC, was usually 98%. Purity can also be assessed by HPLC.

Extracting steroid from media samples

When samples of media were to be analysed for labeled steroid content, the steroid was extracted and purified. 200µl of sample were added to 1ml ethyl acetate, mixed well and centrifuged at 2000rpm (4°C) for 15 minutes. The upper, clear organic phase was transferred to a scintillation vial and dried down (under O_2-free N_2), then re-suspended in 10µl ethanol and stored at -20°C.

Thin-layer-chromatography (TLC)

The TLC plates (Sigma F254) were spotted with steroid in organic phase and run along an organic / inorganic interphase. Labelled steroid was quantified with scintillation or visualisation.

Running the Samples - The purified sample was dissolved in 50µl ethanol containing 0.5mg/ml cold 11-dehydrocorticosterone / corticosterone. The cold steroid amplified the steroid content, aiding rapid visualisation of the run samples under ultra-violet light. Samples were individually spotted 2cm above the base of the plate. It was important for analysis to keep the loading spots as small and

separate as possible. This was achieved by pipetting 3µl a time, ensuring that the spot was dry before the next loading and by keeping a minimum of 1.5mm between spots. Each plate had a control lane, run with cold steroid only. When the plate had dried, it was placed into a TLC chamber which had been equilibriated with 92ml ethanol / 8ml chloroform. The plates were run until the front reached 5cm from the top. They were then allowed to dry.

The running of samples could be checked by visualising the plate under ultraviolet light (254nm). The 11-dehydrocorticosterone ran ahead of the corticosterone.

11-dehydrocorticosterone DPM =
(11-dehydrocorticosterone signal DPM) – (cold control DPM)

corticosterone DPM =
(corticosterone signal DPM) – (cold control DPM)

Reductase conversion =
(corticosterone DPM) as a percentage of total steroid

Reductase conversion =
100 x[(corticosterone DPM)/{(corticosterone DPM) +
(11-dehydrocorticosterone DPM)}]

Figure 4-2: Calculation of steroid reductase conversion

An example of the calculation of the percentage conversion of 11-dehydrocorticosterone to corticosterone by 11β-HSD1. Each steroid has been quantified by the detection of beta-emission particles from the tritium label (decays per minute [DPM]), controlled for the emission levels from unlabelled carrier (cold control DPM). The reductase conversion represents the percentage of original labelled 11-dehydrocorticosterone which has been converted to labelled corticosterone. It is calculated as a percentage of corticosterone (DPM) of total steroid (DPM). Dehydrogenase conversion was calculated as the percentage 11-dehydrocorticosterone of total steroid.

4.2.6. Determination of Neuron / Glial Ratio by Immuno-histochemistry

In order to characterise the cultures, it was important to establish the ratio of neuron to glial cells. This was done by in-situ immuno-histochemistry for cell-type markers. The Vector ABC system was used because of its simplicity and adaptability. The system used a primary antibody against the target protein (raised in a different species) and a secondary antibody against the primary. The secondary antibody was biotinylated and a biotin / avidin complex developed to increase signal. An avidin linked enzyme was then linked with the complex and developed a coloured, stable product from added substrate. This was used to identify the location of the target protein.

An antibody to glial fibrillary acid protein (α-GFAP) (Sigma) was used as a marker for astrocytes and tanocytes. Microtubule-associated proteins (MAP-2) (antibody from Sigma) were used as the cell-markers for the hippocampal, cortical and cerebellar granular neurons.

The markers were applied to separate wells in triplicate. A control well, with no primary antibody was included. The cells were briefly washed with PBS and fixed in fresh 4% paraformaldehyde in PBS (20 minutes at 4°C) (refer to Appendix A for recipes). The cells were then washed in PBS (3x5 minutes) and incubated in 2% H_2O_2 (PBS) for 15 minutes to quench the endogenous catalase and washed. In order to improve antibody penetration, the cells were then incubated in 0.1% Triton X100 in PBS and washed. They were then blocked by incubation for 1 hour at room temperature in 5% bovine serum albumin (in PBS). The bovine serum albumin was drained and appropriate antibody (anti-MAP-2 was applied at 1/1000 dilution and the

anti-α-GFAP at 1/500) applied in blocking solution for 1 hour at room temperature. The cells were washed in PBS.

They were then incubated in secondary antibody (4.5µl /ml) in blocking solution for 1 hour at room temperature and washed 3x5 minutes in PBS. The cells were then incubated for 1 hour in AB (avidin/biotin) (9µl/ml) in PBS, which had been pre-incubated for 1 hour at room temperature. The AB solution was removed and the cells were then washed 3x5 minutes in PBS. The bound antibody could be visualised by incubation in diaminobenzidine tetrahydrochloride (DAB), in accordance with the manufacturer's instructions, or in 3-amino-9-ethyl carbazole (AEC) at twice the recommended working concentration.

The labelled cells were viewed under a phase contrast microscope. This allowed non-labelled and labelled cells to be viewed without counterstaining. The density of cells was heterogenous across the well, being denser towards the centre. A point at the half radius was selected and 100 cells counted, using hand-held counter. The number of labelled cells was identified for each marker (wells in triplicate for each) and a mean value determined.

4.3. Investigation of 11β-HSD1 Activity in Primary Cultured Hippocampal, Cerebellar Granular and Frontal Cortical Cells

4.3.1. Introduction

Cultures of hippocampal, cerebellar and cortical cells were prepared according to the methods described above. There were two main aspects to the experiment: to determine the potential of primary cultures to model activity in expressing, adult cells and determine activity direction; to begin to explore the contribution of glia to *in vitro* conversion. These two aspects could be investigated simultaneously.

4.3.2. Method

Establishing an incubation time - Initially, a time-course of conversion was prepared to establish an end point for comparative cultures (n=1). The cells were treated with 25nM ^{3}H 11-dehydrocorticosterone, ^{3}H corticosterone or control media and incubated for 24 hours. Samples of 200μl media were removed from a 2ml culture at each point.

Pharmacological inhibition - In order to begin verifying that steroid conversion was by 11β-HSD1, a cerebellar granular neuron culture was incubated with carbenoxolone (10μM). Cells were treated as described by Rajan (Rajan et al. 1996), 24 hours before assay (seventh day of culture). Samples were taken 24 hours after the addition of 25nM ^{3}H 11-dehydrocorticosterone. The opportunity was also taken

to explore inhibition by frusemide (10μM) and chenodeoxycholic acid (10μM). This was only tested upon one culture, with each condition in triplicate wells.

Corticosterone / 11-dehydrocorticosterone treatment - Results were generated for each culture type from triplicate wells of three separate cultures derived from separate dams. The cultures were treated in a manner which would establish the required neuron : glia ratio (see below). The seventh day of culture, the cells were treated with a final volume of 25nM ^{3}H-11-dehydrocorticosterone, ^{3}H-corticosterone or control media. The steroid was initially dissolved in ethanol, although the final ethanol was kept to 2% of the added volume. For each culture, triplicate control wells were included, with carrier media (2% ethanol, final volume).

As has been discussed previously, it was thought useful to investigate the significance of glial population in culture for 11β-HSD1 activity. Thus cultures were treated so as to establish low, intermediate and high levels of glia.

Establishing cultures of high neuron:glia ratio - Cultures of low glial population were grown according to the methods described for the steroid conversion experiments (described earlier in this chapter).

Establishing cultures of intermediate neuron:glia ratio - Cultures of intermediate glial proportion were generated by with-holding treatment with the glial proliferation inhibitors AraC and FDU.

Establishing cultures of low neuron:glia ratio - Cultures of high glial population were generated by initially stripping neurons from a freshly plated culture, after a method previously used to examine glucocorticoid activity in glia (Horner et al.

1990). Cells were allowed to plate down for 1 hour. The plates were wrapped to prevent contamination and placed on a bench-top shaker for 1 hour. The cells were then returned to the incubator for 24 hours, at which point neuronal death could be seen. The plates were then washed with media, the culturing media (without glial proliferation inhibitor) was replaced and the cells returned to the incubator. This was a fairly consistent method of stripping but cell populations had to be confirmed.

Statistics - All treatments were performed on cells in triplicate wells and the mean values taken for each triplicate. The time-course and inhibition was performed upon a single culture, but all other experiments were performed in three separate cultures. The graphs show mean values and s.e.m. for these cultures. Analysis is by ANOVA with LSD post-hoc analysis. The analysis was performed using Stastistica software.

4.3.3. Results

Initial verification of activity in cerebellar granular culture - Approximately 45% of the tritium added to a culture was retrieved for analysis.

Time-course analysis of cerebellar granular culture glucocorticoid conversion - Conversion of corticosterone and 11-dehydrocorticosterone in rat cerebellar granular culture showed conversion rates (Figure 4-3) similar to those demonstrated for hippocampal primary cultures (Rajan et al. 1996). Based upon this result, 24 hours was chosen as a representative time point for future assays.

The effects of known 11β-HSD1 inhibitors upon reductase activity in cerebellar granular culture - The most effective inhibitor was carbenoxolone (10μM) (Figure

4-4). The chenodeoxycholic acid was an order of magnitude less effective (under these conditions) and the frusemide intermediate.

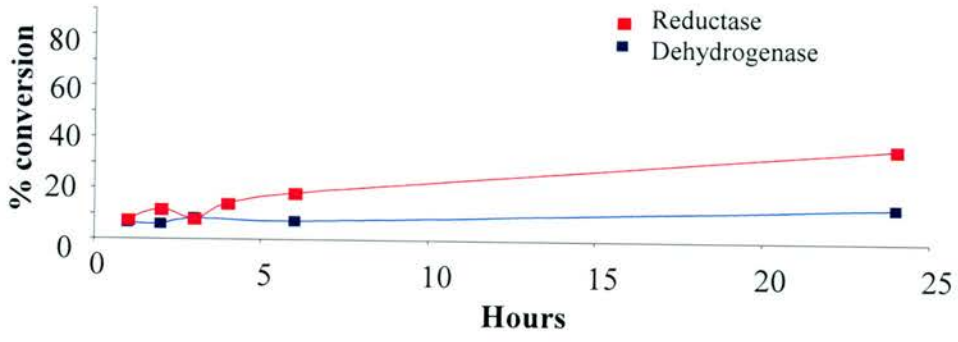

Figure 4-3: Time-course analysis of 11β HSD1-dependent conversion of 11-dehydrocorticosterone or corticosterone by primary cerebellar granular cultures

Cells (seventh day of culture) were incubated with 25nM tritiated 11-dehydrocorticosterone (for reductase direction) or tritiated corticosterone (for dehydrogenase direction) and samples were taken. N= 1 culture with wells in triplicate.

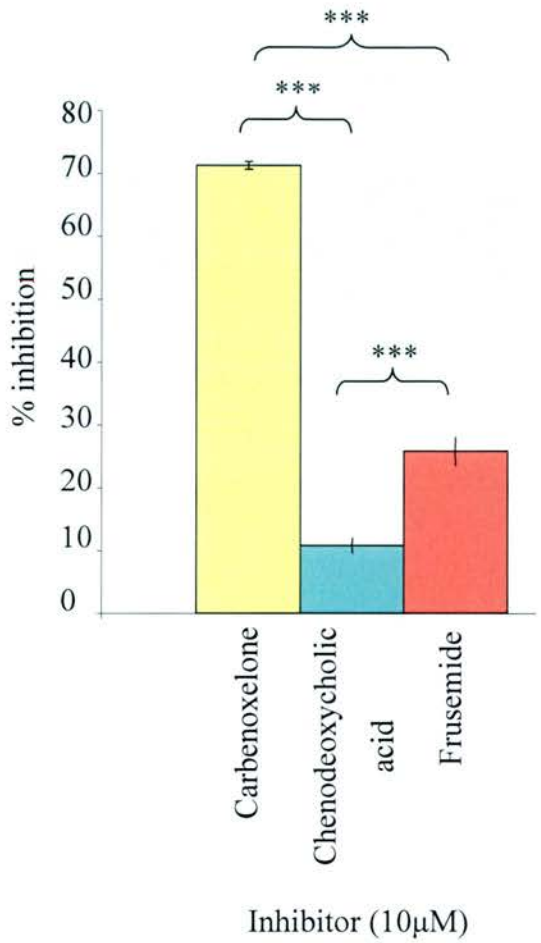

Figure 4-4: Percentage inhibition of 11-dehydrocorticosterone reductase activity cerebellar granular neuron culture by 11β-HSD1 inhibitors

Cerebellar granular neurons were incubated with inhibitor (10μM) 24hrs before the addition of 25nM 11-denhyrocorticosterone. Results are shown for the percentage reductase activity over 24 hours, adjusted for background conversion. N=1 culture with wells in triplicate. *** = $p<0.001$ T-test

Analysis of neuron /glial populations

Culture viability - Healthy mixed populations of glia and neurons were obtained from frontal cortical, hippocampal and cerebellar granular cultures by the methods described above (Figure 4-5). Purkinje neuron populations were healthy but of such low proportions that future metabolic study was abandoned.

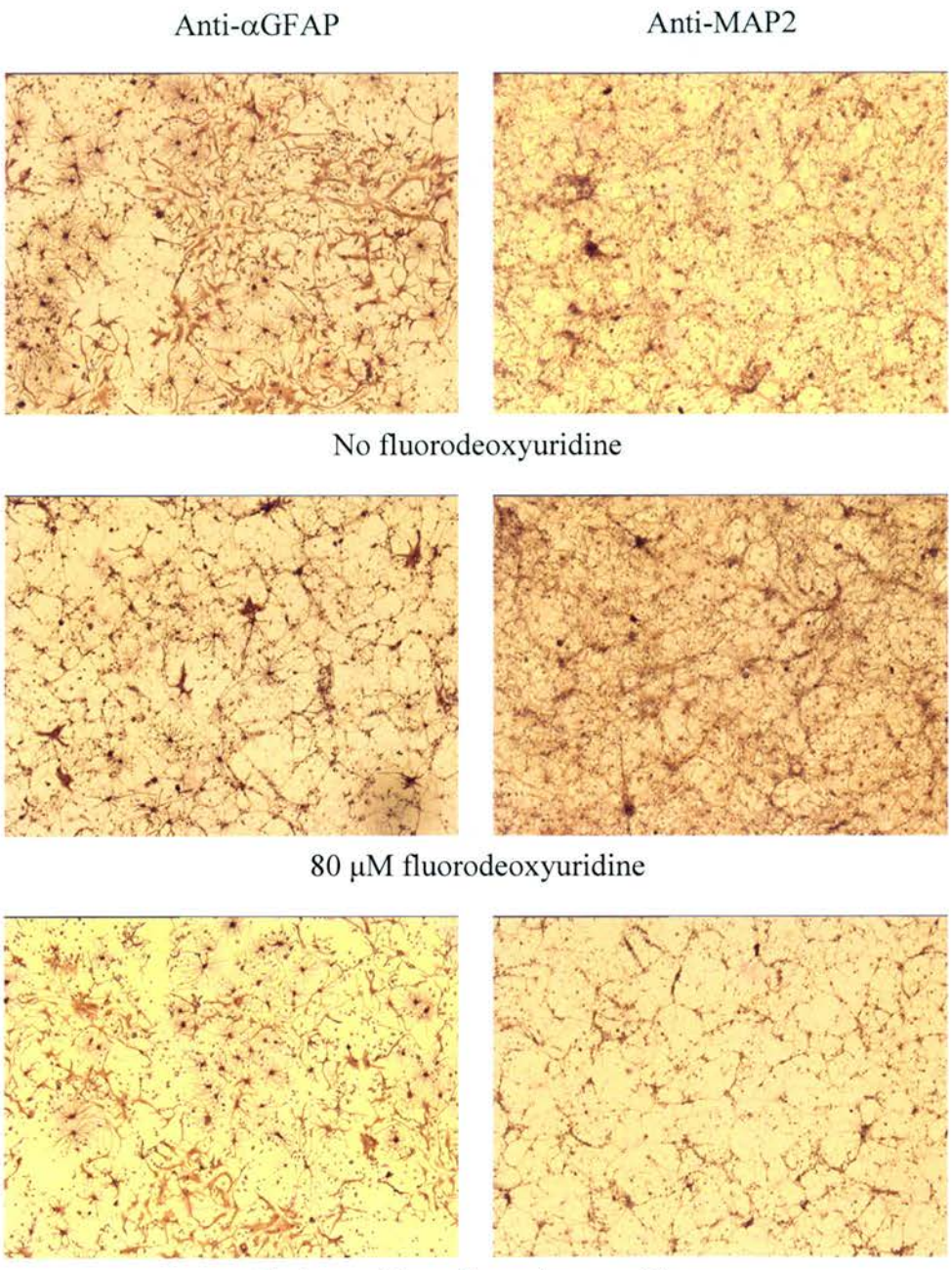

Figure 4-5: Granular neuron primary cultures with adjusted glial /neuronal populations. Immuno-histochemistry for α-GFAP (glial marker) and MAP2 (neuronal marker)

Immuno-labelled cells can be seen in brown (DAB). Non-labelled cells can not be seen. The proportions for hippocampal cultures were very similar. (magnification x500)

Hippocampal culture - The cultures displayed reduction of ^{3}H-11 dehydrocorticosterone, with little dehydrogenation of the ^{3}H corticosterone (Figure 4-6). ANOVA revealed no significant effect of neuron : glia ratio upon oxidation but a significant effect upon reduction ($F_{(2,6)}$=18.16; $p<0.03$).

LSD-post-hoc analysis showed no differences in dehydrogenation. However, the Mixed population showed almost twice reduction of the High Neuron ($p<0.005$) and of the High Glia ($p<0.001$) populations.

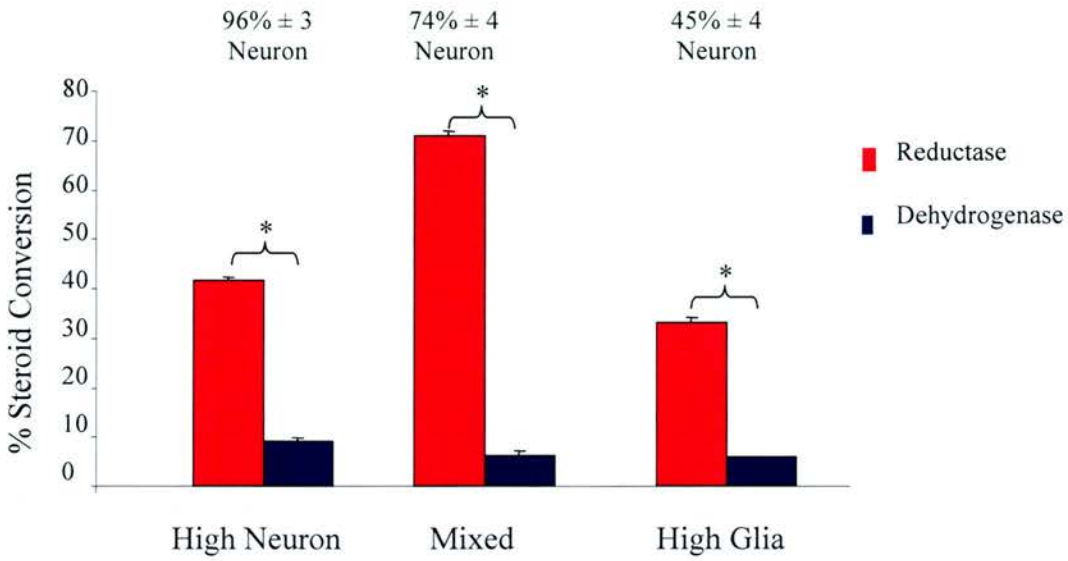

Figure 4-6: Steroid conversion in hippocampal pyramidal mixed cultures of defined neuron/glial proportion

Incubation with 25nM ^{3}H-11-dehydrocorticosterone (reduction) or ^{3}H-Corticosterone (dehydrogenation) for 24 hours. Results are for three separate cultures, with each test in triplicate. Values are adjusted for background. Percentage figures show the mean ± sem proportions of MAP-2 positive cells. * = $p<0.05$ by LSD post-hoc analysis.

Cortical culture - Initial analysis of three cortical cultures (high neuron) had shown negligible 11β-dehydrogenase or reductase activity, despite healthy cultures. Further analysis was therefore abandoned.

Cerebellar granular culture - The cultures displayed 11β-reduction of ^{3}H-11 dehydrocorticosterone, with little dehydrogenation of the ^{3}H corticosterone (Figure 4-7). The ANOVA revealed a significant effect of neuron : glia ratio upon dehydrogenation ($F_{(2,6)}$=6.82; p=0.028) and reduction ($F_{(2,6)}$=45.82; p=0.0002).

LSD-post-hoc analysis showed that the High Neuron culture demonstrated significantly lower dehydrogenation than the Mixed (p=0.029) or the High Glia (p=0.013). In addition, the Mixed population showed twice the 11β-reductase activity of the High Neuron (p<0.0002) and of the High Glia (p<0.0002) populations.

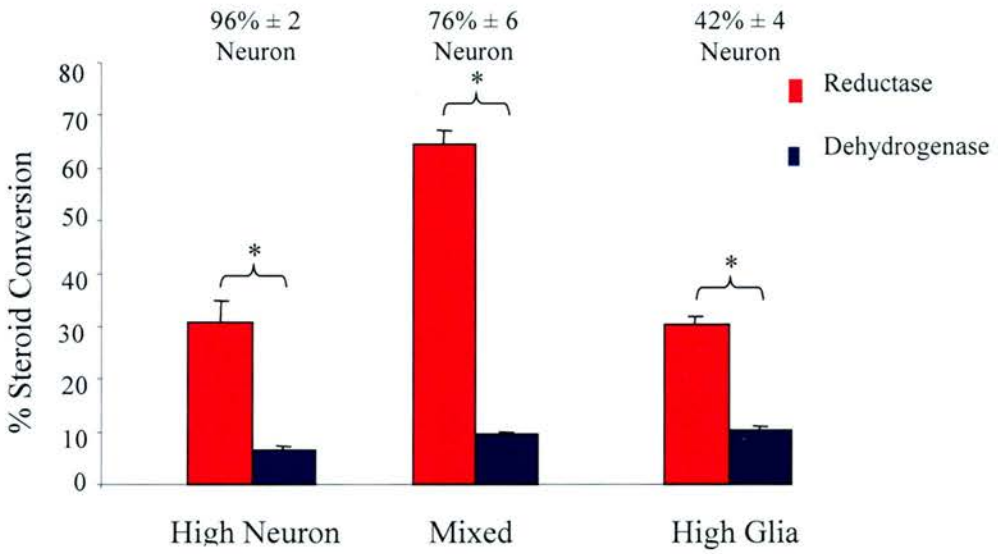

Figure 4-7: Steroid conversion in cerebellar granular mixed cultures of defined neuron/glial proportion

Incubation with 25nM ^{3}H-11-dehydrocorticosterone (reduction) or ^{3}H-Corticosterone (dehydrogenation) for 24 hours. Results are for three separate cultures, with each test in triplicate. Values are adjusted for background. % show the mean proportion of MAP-2 ± sem positive cells. * = p<0.05 by LSD post-hoc analysis

4.3.4. Discussion

Modelling 11β-HSD1 Activity of Cells in the Adult

There was success in demonstrating replication of the previously obtained reductase results for primary hippocampal neurons (Rajan et al. 1996). In addition, a predominantly reductase activity was established for primary cultures of cerebellar granular neurons. However, no activity was found for primary cortical cultures. In-situ-hybridisation of the developing brain had previously demonstrated that at embryonic day 18 (when the hippocampal and cortical cells were taken) the type-1 isozyme had begun to be expressed in the hippocampus but not in the frontal cortex (Diaz et al. 1998). The type-2 isozyme was no longer expressed in either area. The cerebellar region was shown to express the type-1 isozyme at the experimental end-point embryonic day 22.5. It may be assumed that expression continued into adulthood. This may explain the current results and suggests that primary culture of fetal cells is not a good model for 11β-HSD1 activity in the frontal cortex. However, granular neurons appear to provide another primary culture model of activity in adult neurons. As the most abundant neuron in the brain, they also provide a more convenient model.

The Contribution of Glia to Assays of 11β-HSD1 Activity

The hippocampal culture was of a lower glial proportion than that described in the initial report. It was considered that this had perhaps accounted for the slightly lower reductase activities obtained and sparked the investigation of glial proportions. It

was found in both the hippocampal and granular cultures that a mixed population of neurons and glia gave higher reductase activity than either high neurons or high glia.

Attempts were made during the study to identify which cells were expressing the 11β-HSD1 mRNA, using in-situ-hybridisation. This attempt befell technical difficulties, but remains a question to be answered. Further attempts could be made using the technique of combined in-situ-hybridisation for 11β-HSD1 mRNA with immuno-labelling for cell markers, providing a barrier between the chromagen and the emulsion. In addition, development of the non-radioactive in-situ-hybridisation technique or developing a rat monoclonal anti-11β-HSD1 antibody would enable immuno-histochemistry. A double immuno-labelling technique would be much simpler to perform and with the use of fluorescent labels, could be quantitative. Alternatively, the cells of mixed cultures could be sorted with a FACS system and mRNA analysed. This author would avoid looking a bioactivity in sorted cells because of the effect of cellular disruption upon enzyme direction. As discussed previously, 11β-HSD1 as a membrane-bound dimer acts as a reductase, but disruption of this unit favours the dehydrogenase direction (Lakshmi 1985; Maser et al. 2002).

The fact that the mixed cultures (rather than high proportions of either cell-type) gave the higher reductase activity, suggests that the interaction between glia and neurons is important. It is possible that this could have simply been due to a higher biomass and could have been controlled for by adjusting for the protein content of the well. Despite this, observations of the immuno-histochemistry for MAP-2 (refer to Figure 4-5 for examples from cerebellar granular neuron culture) did not suggest

significantly differing neuron numbers between the high and intermediate neuron:glial ratio cultures. It is also possible that the process of stripping the neurons from the 'High Glia' cultures, itself decreased activity and is an artefact.

Future research

High expression of 11β-HSD1 mRNA has previously been described in Purkinje neurons of the cerebellum (chapter 2). It would have been useful to model this activity in primary culture but the attempt was abandoned early in the experiment. Although healthy neurons were obtained, they were too sparse within a mixed culture to confidently attribute any conversion to them. A protocol for enriching Purkinje culture populations has been published, but still reaches levels too low for this type of study (Tabata et al. 2000).

4.4. Summary

The previously found predominant 11β-reductase activity found in primary, rat hippocampal cultures was replicated. In addition, primary cultures of rat cerebellar granule neurons gave comparable predominant reductase activity, suggesting that 11β-HSD1 is acting as a reductase (amplifying intracellular corticosterone) in the adult. There was no predominant dehydrogenase / reductase activity found in cultures of primary cortical pyramidal neurons. This could be attributed to the ontogeny of 11β-HSD1 and 11β-HSD2 at the time of cell isolation. Thus, primary cultures of cerebellar granular neurons may provide another suitable model for future

research in to 11β-HSD1 in the brain. However, other means will have to be found to model 11β-HSD1 in the adult frontal cortex.

In cultures from both the hippocampus and the cerebellum, it was found that modulating the glia: neuron ratio had a significant effect upon 11β-reductase activity, such that a 3:1 ratio gave the highest activity. The question of the relative distribution of enzyme between glia and neurons remains. The results certainly re-enforce that care should be taken when analysing results from mixed cultures. If it were found that glia exhibit significant levels of 11β-reductase activity or influence activity in neurons, this would be of interest to anyone studying developmental / ageing neurology or looking at aspects of plasticity in the adult. The significance of these results to the current thesis will be discussed in more detail in Chapter 5.

Chapter 5

General Discussion

This thesis combined in-vivo and in-vitro techniques to gain insight into the role of 11β-HSD1 in the ageing brain.

5.1. Summary of Findings

Chapter 2 explored molecular aspects of glucocorticoid signalling in the hippocampi and cerebella of young and aged 129/Ola 11β-HSD1 knock-out mice (and their wild-type controls) which we had previously been assessed for hippocampal-dependent learning (Yau et al. 2001). The aged 11β-HSD1 knock-outs were protected from age-related deficits, whereas no differences were found in the young. The in-situ-hybridisation analysis found no reduction in 11β-HSD1 mRNA in the hippocampus or cerebellum with ageing. However, the results confirmed a reduction of GR mRNA in the aged hippocampal CA1 region with no change in any other hippocampal region nor in Purkinje or granular neurons of the cerebellar lobules examined. This was in line with previous reports of the sensitivity of CA1 GR mRNA to ageing effects (Yau et al. 2002; Murphy et al. 2002). Interestingly, there was also a reduction of GR mRNA in the CA1 region in the young knock-out and the difference between knock-out and wild-type was lost in old age. This reduction in GR mRNA could contribute to impaired HPA axis feedback which was seen previously in an 11β-HSD1 knock-out on an MF1 background. The knock-out also showed a reduction in GR mRNA in zones of the granular layer of the cerebellum (not in the Purkinje region) which did not reduce with age. MR mRNA was examined in the hippocampus, where there were no post-hoc differences between groups.

Chapter 3 attempted to confirm the ageing phenotype in an 11β-HSD1 knock-out on a C57BL/6J background. Young and middle-aged knock-out mice were compared with age-matched C57BL/6J controls. Using the Y-maze paradigm to examining hippocampal-dependent spatial learning, no age or genotype-related deficit in exploration strategy, short-term memory or positive drive to explore novel environments were shown. An age-related imapairement of long-term was not clearly demonstrated in the control mice. This may have been due to high stringency of the test (even the young control mice were impaired after a 2 hour inter-trial-interval). However, the middle-aged 11β-HSD1 knock-out mice demonstrated improved long-term memory (individual scores and in the population frequency of the ability to achieve a criterion of performance) compared with the middle-aged control mice. This suggested that some impairement in the ability of the middle-aged control mice was present but not quantified. An alternative (not exclusively) explanation was that some other factor, such as local glucocorticoid during the test, was responsible for the improvement in long-term memory seen in the middle-aged knock-out. In some support of this second mechanism, there was evidence that despite 'forgetting' in the young control mice (a significant decrease seen in performance after the longer interval compared with the shorter), there was no such 'forgetting' in the young 11β-HSD1 knock-out mice. These results suggested that when tested in old age the 11β-HSD1 knock-out on the C57BL/6J background would confirm the ageing phenotype and established the Y-maze as a spatial tool in the laboratory.

In addition, there were attempts made to explore anxiety in the knock-out. This was achieved using the open-field and the elevated-plus-maze. A subtle phenotype of increased anxiety was found, with increased risk assessment in the young 11β-HSD1 knock-out compared with the young controls (elevated-plus-maze) and increased outer-zone activity associated with absence of 11β-HSD1 (open field). Differences were found between the tests and a differential effect of early and late phases of testing. The mice were also tested for cerebellar function on the accelerating rota-rod. There was an impairment shown with ageing and with loss of 11β-HSD1, both in the number of attempts made to achieve performance criterion and in the time spent on the accelerating rota-rod. The results suggested that the age-related motor dysfunction was influenced by something other than current glucocorticoid levels.

The results from the previous two chapters revealed a complex expression of 11β-HSD1 mRNA within the cerebellum and hippocampus and a complex behavioural phenotype which could not simply be explained by a globally lower or higher intracellular corticosterone. It was obvious that the cellular expression of the enzyme and the resulting intracellular level of corticosterone (specific to that cell) were important and attempts should be made to identify the expressing cell-types in regions of interest. This would aid in the dissection of the specific role of local glucocorticoid levels modulated by 11β-HSD1.

The final experimental chapter described attempts to model 11β-HSD1 activity in primary culture of cells from the cerebellum and frontal cortex. This would develop the work performed by Rajan *et al* upon primary hippocampal cells (Rajan et al. 1996). It was found that Purkinje neurons were not suitable for this type of

metabolic modelling and that the ontogeny of enzyme expression in cortical neurons did not make their primary culture a good model of activity in the adult. However, cultures of granular neurons of the cerebellum displayed activity comparable with that of the hippocampal cultures. An observation that the proportion of glia made an effect upon 11-DHC reduction led to the exploration of the role of glia in the conversion seen in mixed cultures. It was found in both hippocampal and cerebellar granular cultures that reductase conversion was enhanced in mixed neuron / glia cultures.

5.2. Interpreting the Spatial Learning Phenotype in Terms of the Molecular Results from Hippocampal In-situ-Hybridisation

This section of the discussion will attempt to combine the spatial, plasma corticosterone and molecular results from Chapters 2 and 3. It should be remembered that these chapters dealt with different strains of mouse and compared young with aged (Chapter 2) and middle-aged (Chapter 3) mice. In addition, the spatial tasks were different, probably involving different HPA axis activation.

Results from this laboratory's *in vivo* and *in vitro* studies, had shown an 11C reductase activity of 11β-HSD type-1 upon 11-dehydrocorticosterone. The results from Chapter 2 had shown relatively high levels of 11β-HSD1 mRNA in the CA3 regions, but expression in the CA1 and DG was limited to cells at the periphery. Cells in the oriens and lacunosum molecularis were also shown to express 11β-HSD1 mRNA. It was possible that a reduction of intra-cellular corticosterone in the CA3 region led to the minor improvement of long-term memory in the young knock-out in Chapter 3 (the non-standard method of analysing this improvement should be treated

with caution). A high MR to GR occupancy in the hippocampus has been shown to improve the persistence of LTP (Korz and Frey 2003). It is also possible that the scattered expressing cells were inter-neurons or glia, in which the reduction of corticosterone had a positive effect upon hippocampal processing. An analysis of 11β-HSD1, GR and MR in the brains of 11β-HSD1 knockout C57BL/6J (sacrificed directly after behavioural testing) would be essential to establish whether the 2 models (11β-HSD1 knockout in the 129/Ola and C57BL/6J background) are directly comparable.

It is notable that despite the improvement in long-term spatial memory in the ageing 11β-HSD1 knock-out (129/Ola), there was not a protection from an age-related down regulation in the GR mRNA of the CA1 region and also that the difference between wild-type and knock-out did not continue into middle age. It is also notable that there was no reduction in 11β-HSD1 mRNA with age. This does not prohibit an age-related reversal of post-translational inhibition by a factor such as IGF-1 (inhibition was previously shown in-vitro in 2S FAZA hepatoma cells (Voice et al. 1996)), which has been shown to decrease in the ageing brain (Lai et al. 2000). Such a mechanism could result in increased 11β-HSD1 enzyme without a change in mRNA. The maintenance of 11β-HSD1 mRNA with age, does indicate that there may still be opportunity for pharmacological intervention in the middle-aged and older adult.

There remain a number of possible mechanisms for the improved age-related cognitive decline seen in the 11β-HSD1 knock-out mice:

1. The absence of reductase activity in the CA3 could be neuroprotective in that region or the expressing cells dispersed around the CA1 and DG could be acting in a paracrine manner, reducing the active glucocorticoid in non-expressing cells.

2. The reduction in GR mRNA in the CA1 of the knock-out young, could be neuroprotective. This reduction could be in response to high circulating corticosterone. This explanation would require some other neuroprotective effect because high circulating corticosterone is usually associated with deficits. A reduction in the GR:MR signalling ratio may attenuate neurotoxicity (Sousa et al. 1999; Elliot and Sapolsky 1992) or damage from ischaemic insult (Macleod et al. 2003; McIntosh et al. 1998). It may also result in a protection of pyramidal cell excitability.

3. There could be a developmental effect of the absence of 11β-HSD1 (pre- and post-partum) which down-stream has neuroprotective properties.

4. 11β-HSD1 could be expressed in interneurons in the polymorphic region, the molecular regions and the oriens. As such, they could be modulating neurotransmitter control of hippocampal excitability.

5. 11β-HSD1 could be expressed in astrocytes. As such, it could be involved in the supply and regulation of glucose (and other fuels) (Horner et al. 1990; Virgin, Jr. et al. 1991; Wender et al. 2000). It could also be involved in the clearance of excitotoxins from the synapse (Hardin-Pouzet et al. 1996).

5.3. Interpreting the Anxiety Phenotype

The role of glucocortioids in hippocampus-mediated anxiety is complex. Lesions of the hippocampus have been correlated with reduced anxiety (Bannerman et al, 2002). Both MR and GR have been implicated in anxiety-like behaviours (Tronche et al. 1999; Calvo and Volosin 2001; Smythe et al. 1997). In general the data from both the open-field and elevated-plus-maze suggested a mild increase in anxiety-related exploration. The increased risk assessment in the elevated-plus-maze suggested an influence of the high circulating plasma corticosterone (Rodgers et al. 1999) These correlations suggest that overall, the increasing plasma corticosterone in 11β-HSD1 non-expressing cells of the amygdala or hippocampus of the knock-out is responsible for the indicators of increased anxiety. If the molecular results from the 129/Ola are found in the C57BL/6J, it is possible that a increased MR/GR signaling ratio (at intermediate corticosterone levels) in regions such as the CA3 are increasing anxiety (Smythe et al. 1997). To this author's knowledge, there have not been significant studies of direct quantitative correlations between corticosteroid receptor expression in the hippocampus and anxiety-related behaviour.

5.4. Interpreting the Motor Learning Phenotype

There has been little research into corticosterone effects upon rota-rod performance. It is notable that a somatostatin knock-out with increased plasma corticosterone performed poorly in a similar task to the one described here (Zeyda et al. 2001). In that study, the effects were attributed to developmental effects, but the current data suggests that it could have been due to the increased circulating corticosterone.

Indeed there was a negative correlation between time spent on the Rota-rod with basal plasma corticosterone across the groups and the young. A positive role for cerebellar glucocorticoid (and perhaps 11β-HSD1) has been suggested in a form of learning previously (Johnston et al. 2002) and it is possible that the same core mechanism is involved. There was no age-related change in 11β-HSD1 mRNA in the cerebellum, but there was a decrease in GR mRNA in some regions of the granular layer with a loss of 11β-HSD1, despite no correlation between the level of GR mRNA and 11β-HSD1 mRNA.

The results from Chapter 4 and the identification of predominant reductase activity in primary cultures of cerebellar granular neurons is of interest. This will provide another model of enzyme activity in the brain. The results do suggest that the enzyme is acting as a reductase *in-vivo*. This would mean that corticosterone levels would be higher in 11β-HSD1 expressing cells in the control cerebellum than in correlating cells in the 11β-HSD1 knock-out. Thus this variation may accunt for the impaired performance of knock-outs in the Rota-rod test. The functional significance of enzymes direction of activity within the *in vivo* model remains to be explored.

5.5. Interests for the Future

The analysis of the behavioural results has had to account for the elevated levels of corticosterone seen in the 11β-HSD1 knock-out. It would be useful in future investigation, at the molecular and behavioral level, to investigate the significance of elevated plasma corticosterone within this model. As discussed previously, it would

be useful to establish plasma corticosterone values at the time of behavioural testing, perhaps by immediate sampling. In addition, it would be useful to fix levels of plasma corticosterone through adrenalectomy and controlled replacement. A brain specific knock-out would control for interactions such as liver and adipose effects upon blood lipid and glucose profiles. An inducible knock-out could account for potential developmental effects. A non-pituitary knock-out would help to control for loss of HPA control at the pituitary and explore effects above this. The behavioral experiment had to be ceased when the mice were only middle-aged, because of time constraints. It is interesting to note that they have now been re-tested in old age by other in the laboratory and the results from the Y-maze are consistent with those found in this thesis when tested at middle-age.

There were attempts made, during the course of this study, to identify the cell types expressing 11β-HSD1 mRNA. These attempts were not successful. It would be worth persisting with the development of this technique. Establishing expressing cell types would have been the first stage of a series of useful experiments. The hippocampal distribution of 11β-HSD1 mRNA in the mouse bore some resemblance to that of IGF-1 and of CRH. It would be interesting to explore possible co-localisation with these and other factors, in order to explain the behavioural phenotype in the young and the aged animal. The development of a good monoclonal 11β-HSD1 antibody would make this process much simpler and could be combined with RNA-based techniques to give enhanced information. In light of the general theme of the importance of cellular rather than tissue-level

corticosterone, it was important to the current thesis and should be in further studies, to preserve that level of investigation.

It would be interesting to explore the role of 11β-HSD1 in behaviour further. A more detailed mapping of message and protein in the rodent brain would be required (e.g. sub-regions of the amygdala, ventral and dorsal hippocampus). If the 11β-HSD1 is affecting MR/GR activation, will it have an effect upon conditional learning? It is certainly essential to explore the role of glucocorticoids and 11β-HSD1 in cerebellar function. In order to further investigate the role of the enzyme in ageing of the hippocampus, it would be useful to look at some of the markers of ageing such as reactive gliosis, synaptic bouton density, LTP and of excitatory amino acid toxicity. In light of the expression of 11β-HSD1 around the DG, a BrdU integration study looking at neurogenesis would reveal potential effects there. Of particular interest in the study of ageing and considering the role of 11β-HSD1 in the periphery, it would be fascinating to look at the effects of the enzyme upon brain energetics.

In summary, this thesis has confirmed the ageing phenotype of the 11β-HSD1 knock-out and shown that protection from glucocorticoid-associated hippocampal damage occurs from middle-age. In the young there is a subtle increase in some anxiety behaviours and a behavioural activation in the open-field; and an inhibition of a motor-learning task. There is no change in 11β-HSD1 mRNA in the ageing hippocampus or cerebellum. The young knock-out demonstrates reduced GR mRNA in areas of the hippocampus and cerebellar granular layer. In general, there is a dissociation between GR mRNA in the hippocampus, glucocorticoid-related

behaviours and plasma corticosterone. Finally, it was shown that primary culture could be used as a model of 11-dehydrocorticosterone reduction in the cerebellar granular layer and that glia may play a significant role in 11β-HSD1 biochemistry (at least *in vitro*).

Appendices

Appendix A

A.1. Solutions prepared for mRNA in-situ-hybridisation in fresh frozen tissue:

General solutions

Box buffer		50% deionised formamide, 20% 20xSSC, 30% DEPC-treated water. 20 – 25mls per box.
Deionised formamide		150ml formamide (Sigma-Aldrich Company Ltd., UK) mixed with 15g Amberlite mixed bed ion exchange resin (BDH [Merck] Ltd., UK) for 1 hour at room temperature, in the fume hood. Then double filtered (Whatmann No 1), aliquoted and stored at – 20°C, protect from the light.
DEPC-treated water		In the fume hood, double-distilled water was treated with DEPC (Sigma-Aldrich Company Ltd., UK) (1 drop per 100ml added in the bottle and left for 2 hours). The DEPC was inactivated by autoclaving
DTT 1M		(Sigma-Aldrich Company Ltd., UK) Prepared in DEPC-treated water. Aliquoted and stored at -20°C.
EDTA (disodium EDTA·2H_2O) 250mM		(Sigma-Aldrich Company Ltd., UK) Prepared with double distilled water, pH 8.0 and autoclaved.
Ethanol in 0.3M ammonium acetate		50%, 70% and 90% in 0.3M ammonium acetate (Sigma-Aldrich Company Ltd., UK) in distilled water.

LB (Lauria-Bertani) broth	1 ltr	10g Bacto tryptone (BDH [Merck] Ltd., UK), 5g Yeast extract, 5g NaCl. Autoclave immediately.
LB agar with ampicillin plates		LB broth with 15g/L agar. Autoclaved. Cooled to 55°C, 50μg/ml ampicillin then added and the plates poured.
NaCl 5M		(BDH [Merck] Ltd., UK) Prepared in DEPC-treated water.
Paraformaldehyde (4%) in 0.1M phosphate buffer	1 ltr	(Fisher Scientific, UK) (3.12g NaH_2PO_4, 11.28g Na_2HPO_4, 1l DEPC-treated water). Heated to 80°C (stirring) in the fume hood, 40g papraformaldehyde added, covered and stirred for 1 hour. Cooled to 4°C.
Phosphate buffered saline *PBS x 10*	1 ltr	40.8g NaCl, 14.53g Na_2HPO_4 ($2H_2O$), 1.2g KH_2HPO_4, 1.2g KCl. Prepared with DEPC-treated water and autoclaved.
RNase buffer		10mM Tris-HCl (pH 7.5) (BDH [Merck] Ltd., UK), 1mM EDTA (pH 7.5) (Sigma-Aldrich Company Ltd., UK) , 0.5M NaCl in distilled water.
SSC x 20	0.5 ltr	87.7g NaCl, 44.1g trisodium citrate hydrated (Sigma-Aldrich Company Ltd., UK). Prepared with DEPC-treated water, pH to 7.0 and autoclaved.
Triethanolamine 0.1M (pH8.0)	1 ltr	13.3ml triethanolamine (Sigma-Aldrich Company Ltd., UK) in DEPC-treated water. pH and autoclaved.
Tris 1M		(BDH [Merck] Ltd., UK) Prepared with DEPC-treated water, pH 7.5 and autoclaved.

Hybridisation Buffers (10ml)

	2 x Prehybridisation Buffer	**2 x Hybridisation Buffer**
Reagent	Volume of reagent	
DEPC-treated water	5.88ml	6.68ml
5M NaCl	2.4ml	2.4ml
1M Tris (pH 7.5)	200µl	200µl
50 x Denhardts (Sigma-Aldrich Company Ltd., UK)	400µl	400µl
250mM EDTA	80µl	80µl
50mg/ml Salmon sperm DNA (Sigma-Aldrich Company Ltd., UK)	1ml	200µl
Dextran sulphate (Sigma-Aldrich Company Ltd., UK)	40µl	40µl
Yeast tRNA (Gibco BRL [Life Technologies Ltd.]		2g

A.2. Procedures

Subcloning

Making cells competent - 0.5ml of bacteria was grown in 50ml LB until late log phase (overnight) and spun at 7k rpm for 5 minutes. The pellet was resuspended in 20ml of cold CaCl (0.1M) and left for 2 hours. The tube was spun at 7k rpm (5 minutes) and the pellet resuspended in 2ml of cold CaCl (0.1M). The competent cells were stood on ice for 2 hours.

Cell transformation - 50µl of these competent cells were transferred into a tube on ice. The DNA (0.5µl) was added and the tube flicked to mix. This was left on ice (30 minutes), heat-shocked at 42°C (2 minutes), then returned to ice (2 minutes). The cells were plated out on an LB-ampicillin plate and incubated for 16 to 20 hours.

DNA extraction - A single colony was grown in 2ml of LB-ampicillin for 6 to 8 hours and then diluted in 500ml to be grown overnight. This culture was then spun at 6k rpm (4°C for 5 minutes). The pellet was resuspended in 12 ml GTE (50mM glucose, 10mM EDTA, 25mM Tris pH8.0, water) and 24ml of freshly prepared alkaline SDS added (0.2M NaOH / 1%SDS). This was mixed well and placed on ice (10 minutes). 16ml of cold potassium acetate (5M in 11.5% Glacial acetic acid in water) was added, gently mixed and returned to ice (10 minutes). The tube was spun at 6k rpm (4°C, 10 minutes). The supernatant was then filtered through sterile gauze, 32ml of isopropanol added and the tube left at room temperature (30 minutes).

The tube was then spun at 10k rpm (3 minutes). The pellet was air-dried, resuspended in 2.2ml TE and prepared for separation through a CsCl-ethidium bromide gradient. 3g of CsCl is added, with 200µl ethidium bromide (for visualisation) and the volume transferred to a small sealing ultracentrifuge tube. The tube was topped with TE / CsCl (be sure to balance tubes) and the tube heat-sealed. The tube was then spun at 7k (16 to 20 hours).

Purification of DNA - The DNA band (stained with ethidium bromide) was extracted and transferred to a glass culture tube. The ethidium bromide was extracted by repeated washes in isopropanol (if the CsCl came out of solution a small volume of TE could be added). The CsCl was then removed by dialysis overnight against 3 changes of TE.

The resulting DNA was quantified (Pharmacia Biotech Gene Quant) and the quality tested, before further purification.

Estimation of DNA concentration and integrity - DNA was analysed using a Gene Quant analyser, according to the system protocol, at a 1/50 dilution in water and results given as concentration of DNA, level of salt and protein contamination.

The DNA integrity could also be checked on a 1% agarose gel.

Cutting Template

At this point the DNA was circular and had to be linearised before transcription. It was also useful to initially confirm the insert by a double digest at specific points encompassing the insert and visualisation on an agarose gel (refer to figure 2-1).

Template digestion was achieved by incubation of 5µl DNA, 10µl buffer (Promega), 3µl restriction enzyme (Promega) in 100µl final volume with DepC water, for 2.5 hours (37°C) (refer to Appendix B for details). The tube was then transferred to ice and the product checked by densitometry. The DNA could be further cleaned using the Hybaid Recovery DNA purification kit II (RY17050), based upon silica gel column filtration and performed according to the manufacturer's instructions. The resulting DNA could be checked in the same manner as the un-cleaned template.

Generation of ^{35}S-labelled Riboprobes

Reagent	**Volume (µl)**	**Notes**
5 x transcription buffer (Promega)	2	
10mM ATP (Promega)	0.33	
10mM CTP (Promega)	0.33	
10mM GTP (Promega)	0.33	
200mM DTT (in DepC H_2O)	0.5	Freshly made
Rnase inhibitor (Promega)	0.4	
DNA Template	1	
35S-UTP (Amersham Pharmacia Biotech UK)	4	
RNA polymerase (Promega)	1	T3 / T7 / SP6

Transcription - The eppendorf was incubated at 37°C (40°C for SP6) for 60 to 90 minutes, then 1µl of Dnase1 (Rnase free) was incubated with the mix (15 minutes) to digest the DNA template. The RNA was purified by Nick column into 400µl TE buffer.

Checking riboprobes on urea / acrylamide gels - Gel preparation: 3.6g urea (Sigma), 1.32ml 40% acrylamide (Sigma), 1ml 10xTBE and take to 10ml with distilled water. 100µl of ammonium persulphate solution (Sigma) and 10µl of TEMED (N,N,N',n'-tetramethylethylenediamine) (Sigma) were added to polymerise the acrylamide and pour the gel immediately between 2 clean, sealed plates. When set, the gel was placed in the running apparatus and loaded with 1xTBE. Volumes of probe ($1x10^6$ counts) were taken and denatured in denaturing loading buffer at 80°C for 5 minutes. The wells were rinsed with 1xTBE and the samples loaded directly. The gel was run at 15mA until the blue front had run. Once run, the gel was wrapped in Saran-wrap (or similar) and visualised on a phorphorimager or against β-max for ^{35}S film. A good probe appeared as a tight single band.

Tissue preparation for fresh frozen brain sections

Sections were placed, frozen, into ice-cold paraformaldehyde (inhibiting RNase activity) for 10 minutes. The sections were then washed in 1 x PBS twice for 5 minutes. They were then incubated for 10 minutes in the triethanolamine to which had been added 0.25ml acetic anhydride (to increases the signal to noise ratio) immediately as the sections were placed into the trough. Sections were then washed in 1xPBS for 5 minutes and then dehydrated through an ethanol/water series (70%,

80%, 95%) for 2 minutes each. Slides were left to air dry for a maximum of 15 minutes.

Subbing of slides

Glass microscope slides (BDH) were placed in 0.2M HCl (DEPC-treated water), DEPC-treated water and acetone (BDH) (each for 3 minutes). The slides were then dried in a 50°-60°C oven and then dipped in subbing solution (0.03% sodium azide, 0.15% gelatine solution in DEPC-treated water, heated to 50°C until dissolved, then passed through a Whatman n°1) for 5 minutes and dried in the oven overnight. The slides were then dipped in poly-L-lysine (Sigma) (100mg/500ml DEPC-treated water) for 10 minutes. They were oven dried and packaged in aluminium foil.

Post-hybridisation washes

The excess RNase buffer was drained and the slides placed in a rack. The slides were run through a series of 1 hour, SSC washes: 2xSSC (room temperature); 0.1xSSC (60°C); 0.1xSSC (60°C down to room temperature); 50% ethanol in ammonium acetate (room temperature); 80% ethanol in ammonium acetate (room temperature); 95% ethanol in ammonium acetate (room temperature).

Gene	Probe	Vector	Probe length (kb)	Restriction enzyme	RNA polymerase
Rat 11β-HSD1	Antisense	Bluescript Ks	0.6	Sty I	T3
Rat 11β-HSD 1	Sense	Bluescript Ks	0.6	Sty I	T7
Mouse 11β-HSD1	Antisense	PCR ™ 11	0.45	Not I	SP6
Mouse 11β-HSD1	Sense	PCR ™ 11	0.45	Kpn I	T7
Mouse 11β-HSD2	Antisense	pGEM3	0.66	NotI	T7
Mouse 11β-HSD2	Sense	pGEM3	0.66	NcoI	SP6
GR	Antisense	pGEM3	0.67	Ava I	T7
GR	Sense	pGEM3	0.67	EcoRI	T7
MR	Antisense	pGEM4	0.5	Hind III	SP6
MR	Sense	pGEM4	0.5	EcoRI	T7

Appendix C

This appendix contains information for the preparation of media for the culture of rat neurons from different regions of the brain (refer to chapter 4). Reagents are arranged according to their order in the chapter. Solutions were prepared in aseptic conditions and filtered where appropriate.

C.1. Primary hippocampal, pyramidal neuron media

Dissection Media / Hanks Hepes

(Enough for 2 litters)

50ml Hanks Balanced Salt Solution (No Ca or Mg) (HBSS) (Gibco 14170-088), 750µl HEPES (Sigma H0887), 1ml antimycotic/antibiotic mix (Gibco 15240-096)

Plating Media

50ml Neurobasal-A Medium ((Gibco 0888-022), 5ml Foetal Calf Serum (FCS) (Hyclone SH30070.03), 50µl Gentamicin (Gibco 15710-031)

Chemically Defined Media

The aqueous and ethanolic stocks (listed below) were prepared previously, aliquoted and stored at -20°C. In the interests of convenience, the media was prepared at 500ml a time. Fresh stock was made in HBSS. All stocks were double filtered before adding to Neurobasal-A Medium.

Ingredient	**Sigma Reference**	**Final μg/ml**
Aqueous stock		
Biotin	B4501	0.1
L-Carnitine	C0283	2
Ethanolamine	E9508	1
D(+)-galactose	G0625	15
Putrescine	P7505	16.1
Selenium	S1382	0.016
Ethanolic stock		
Linoleic Acid	L1376	1
Linolenic Acid	L2376	1
Retinyl Acetate*	R7011	0.1
D.L-alpha-tocopherol	T3251	1
D.L-alpha-tocopherol Acetate	T3001	1

- dissolved in 50% vol water, then 50% vol alcohol.

	Sigma Reference	Final μg/ml
Add fresh		
Bovine Serum Albumin	A7030	2500
Catalase	C40	2.5
Glutathione	G6013	1
Insulin	I5500	4
Superoxide Dismutase	S2515	2.5
Transferrin	T5391	5
Triodothyronin	T2877	0.002

C.2. Primary, cortical pyramidal neuron media

Dissagregation media

Trypsinisation solution - 2ml DNAse (2,500iu / ml in HBSS), 8ml Trypsin (1.125mg/ml HBSS)

Trypsin inhibitor - 9mg in 10ml HBSS.

Trituration solution - 2ml DNAse, 8ml HBSS.

C.3. Primary, cerebellar granular neuron media

Dissection media

50ml Earle's Balanced Salt Solution (EBSS) (Gibco 14150), 1ml Antibiotic/antimycotic /mix (Gibco 15240-096)

Disaggregation media

Solution 4 - 150mg/ml Bovine Serum Albumin (BSA) fraction V (Sigma A-8022), 0.7M Glucose (0.13g/ml), 75mM $MgSO_4.7H_2O$ (18mg/ml)

Trypsinisation buffer - 10ml EBSS, 200µl Solution 4, 100µl Trypsin Type 1 (Sigma T-8003) 25mg/ml

Trypsin inhibition buffer - 9ml EBSS, 200µl Solution 4, 1ml Soybean Trypsin Inhibitor (Sigma T-9003) 4mg/ml. 200µl Deoxyribonuclease I (DNaseI) (Sigma D-4527) 200U/ml, 200µl $MgSO_4.7H_2O$ (300mM)

Disaggregation buffer - 8ml EBSS, 200µl Solution 4, 1ml Soybean Trypsin Inhibitor, 600µl DNaseI

200µl $MgSO_4.7H_2O$ (300mM).

Gradient buffer - 10ml EBSS, 400mg BSA Fraction V, 200µl Solution 4

Warming the EBSS, to 37°C, before addition of the BSA assisted solution.

Plating media

50ml Modified Eagle's Medium with Earle's Salts without L-Glutamine (Gibco 21090), 5ml Foetal Calf Serum (Hyclone SH30070.03), 150µl Gentamicin (Gibco 15710-031), 200µl KCl (2.45M).

C.4. Stripping corticosterone from Foetal Calf Serum

100mls foetal calf serum was stirred overnight (4°C) with: 0.25M sucrose (BDH); 1.5mM $MgCl_2$ (Sigma); 10mM Hepes (BDH); 0.25% activated charcoal (Sigma); 0.0025% Dextran T70 (Pharmacia). This was then spun (500g for 10 minutes) and the supernatant removed and re-spun. Under the culture hood then supernatant was then double passed through a series (5µm, 1.2µm, 0.45µm, 0.2µm) of sterile filters (Sartorius).

C.5. Conversion of corticosterone to 11-dehydrocorticosterone

C Buffer

8% glycerol, 150mM NaCl, 1mM EDTA, 50mM Tris, double distilled H_2O, pH 7.7.

125µl ^{3}HB is 'blown down' to remove all solvent (essential) and reconstituted in 50µl ethanol. This is then added to 200µl 25mM NAD (Sigma N1636), 300µl homogenised rat placenta ($1^1/_2$ placenta in 1ml C buffer), 4.45ml C buffer (pH 7.7).

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